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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Nitric Oxide (NO) is a potent bioactive molecule produced in the presence of endothelial (e), neuronal (n) and inducible (i) types of NO synthase (NOS) enzymes. We discovered that IL-2 therapy-induced capillary leakage was due to iNOS induction leading to overproduction of NO and its derivative peroxynitrite. We also found that tumor-derived NO promoted mammary tumor progression in mice. eNOS expression by tumor cells was positively correlated with metastasis in spontaneous C3H/HeJ mammary tumors and transplants of two clonal derivatives of a spontaneous tumor differing in metastatic phenotype: highly metastatic C3L5 and weakly metastatic C10 cell lines. These cell lines also exhibited a parallel difference in invasiveness *in vitro* and growth rates as well as angiogenic abilities *in vivo*. A causal relationship between NO production by the tumor cells and invasive, migratory and angiogenic abilities was demonstrated. Invasion stimulation by NO resulted from an upregulation of MMP-2 and a downregulation of MMP inhibitors TIMP-2 and TIMP-3. Migration stimulation by NO resulted from activation of guanylate cyclase and MAP-kinase pathways. Thus NOS inhibitors should have a valuable therapeutic role for blocking multiple steps in mammary tumor growth and metastasis such as tumor cell migration, invasion and angiogenesis.

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5. INTRODUCTION

Overall objectives of the present project were to identify the precise role of nitric oxide (NO) in mammary tumor progression, using a C3H/HeJ mouse mammary tumor model developed in our laboratory. This model includes spontaneous tumors as well as their clones which vary in their ability for spontaneous metastasis.

Biology of NO

Following the discovery in 1987 (1) that NO accounts for the full biological activity of a vasodilator factor initially named in 1980 as the "endothelium-derived relaxing factor" produced by endothelial cells (2), research on the biology of NO has grown exponentially for many years. This molecule has since been shown to be produced by many other cells in the body, providing additional physiological functions such as inhibition of platelet aggregation, modulation of neurotransmission and mediation of cytotoxic function of macrophages against microbes, parasites and tumor cells (3-8). Sustained high levels of NO produced at the sites of inflammation can also mediate pathological injuries (9). In recognition of the significance of the rapid growth of research on NO, in 1992, NO was named as the "molecule of the year" by the journal Science and in 1998, the Nobel Prize was awarded to Robert Furchgott, Louis Ignarro and Ferid Murad for their fundamental work on the biology of NO.

NO is produced by the conversion of the amino acid L-arginine to L-citrulline by a family of enzymes known as NO synthases (NOS). Three isoforms of NOS have been identified so far: endothelial type or eNOS is a constitutive form present in endothelial cells, myocardial cells and other cells inclusive of certain tumor cells; neuronal type or nNOS is also a constitutive form present in the central nervous system neutrons, cells of the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells as well as in tumors of the central nervous system; inducible type or iNOS is usually induced by certain inflammatory cytokines (e.g. IFN-γ, TNF-α) or bacterial products (e.g. LPS) in macrophages, hepatocytes, chondrocytes, endothelial cells and certain tumor cells (10-14). The constitutive forms are Ca^{++} and calmodulin-dependent whereas the inducible form is Ca^{++} and calmodulin-independent. Genes for all the isoforms have been cloned in numerous species (15,16) and disrupted in mice to show that none of the disruptions were embryo-lethal but had pathological effects consistent with known biological functions of NO. For example, eNOS knockout mice are hypertensive (17) because of the loss of vaso-relaxant function of NO; iNOS knockout mice are susceptible to infection and show poor macrophage cytotoxicity against parasites and tumor cells (18), consistent with NO-mediated macrophage defence; nNOS knockout mice (19) show hypertrophic pyloric stenosis, consistent with NO-mediated relaxation of pyloric sphincter muscle. nNOS -deficient males, in addition, show abnormal sexual behavior (20) because of aberrant neurotransmission.

NO is a free radical capable of crossing the cell membrane and reacting with other molecules. Most physiological functions of NO are mediated by increases in intracellular cGMP (21,22), whereas antibacterial, antiparasitic and antitumor functions of macrophage-derived NO have been ascribed to the inhibition of mitochondria respiration and DNA synthesis in target cells (23).

Constitutive production of NO occurs in cells at low to moderate levels, and the resulting bioactivity is short lived (T½ = few seconds) and short-range in nature. On the other hand, induced production of NO can be sustained at high local levels for a longer duration if the inducer molecules, e.g. inflammation-associated cytokines are produced in a protracted manner. This often leads to pathological consequences, resulting from NO reaction products. NO reacts with molecular oxygen, transition metals and superoxide to form intermediates which can cause cellular injury. For example, NO reacts with superoxide to make peroxynitrite, which can cause DNA damage (24).

Role of NO in tumor progression

It has been recognized for some time that chronic NO production is genotoxic and thus potentially carcinogenic (24). Recent studies, including our own (25) have revealed that tumor or host-derived NO can profoundly influence tumor progression in a positive or negative manner depending on the circumstances, and that in a large panel of well-established tumors, which have been examined so far, NO usually promotes tumor progression. Elevated serum NO levels have been observed in many cancer patients (26) indicating that tumor cells or host cells serve as the additional source of NO in these patients. A high expression of active NOS enzymes in tumor cells (27,28,31-34), endothelial cells in tumor vasculature (28) or tumor-infiltrating macrophages (29,30,32,34) has been positively correlated with the degree of malignancy in human cancers involving a large number of tissues: cancers of the reproductive tract (uterus, ovary) (27), central nervous system tumors (28), breast cancer (29,34), gastric cancer (30), cancer (squamous cell carcinomas) of the head and neck (31), prostate cancer (32) and lung cancer (33). However, the underlying mechanisms remained unexplored. Unexpectedly an inversion of this relationship was reported for human colonic tumors (35). This anomaly has been explained in a recent study (36) showing that the highest expression of active iNOS was noted in human colonic adenomas prior to their progression into carcinomas, consistent with the hypothesis that endogenous NO promoted the transition of adenomas into carcinomas by promoting mutations and stimulation of angiogenesis. Indeed, these authors have shown a positive association between certain p53 mutations and iNOS expression in human colonic tumors indicating the promoting role of NO in colonic carcinogenesis (37). A positive correlation between NOS expression or NO production and tumor progression has also been detected in experimental tumor models in the mouse (38) and the rat (39).

A direct evidence for a stimulatory role of NO in tumor progression came from our own findings in a murine mammary adenocarcinoma model that treatments with either of two NOS inhibitors N^G-methyl-L Arginine (NMMA)(40)and N^G-nitro-L-arginine methyl ester (L-NAME)

(41) reduced the growth of the primary tumors and their spontaneous lung metastases in mice transplanted with the C3L5 mammary tumor line (reviewed in 25). Similar findings were reported with L-NAME therapy in a rat colonic adenocarcinoma model (39). In support of these results, engineered expression of iNOS in a human colonic adenocarcinoma line resulted in an increased growth rate and vascularity of tumors following transplantation in nude mice (42). In contrast with these results, engineered overexpression of iNOS in an iNOS deficient murine melanoma line (43,44) or a human renal carcinoma line (45) suppressed tumorigenic and metastatic ability of tumor cells *in vivo* because of NO-mediated cytostasis and apoptosis (43,44). Two explanations may be offered for these apparently conflicting results: First, very high NO levels (such as those produced by the iNOS-transduced murine melanoma line) (43,44) can be detrimental to tumor cell survival; for example the iNOS-overexpressing melanoma line had poor survival in the absence of NOS inhibitors *in vitro* and *in vivo* (44). Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis because of their genetic or biochemical makeup. For example, it has been suggested that the functional status of the tumor suppressor gene p53 dictates susceptibility (if functional) or resistance (if non-functional) to NO-mediated cytostasis or apoptosis (46,47). This suggestion was based on the following findings: iNOS transfected tumor cell lines fell into two distinct categories. Those expressing functional wild type p53 were vulnerable to NO-mediated cytostasis because of an accumulation p53 protein induced by endogenous NO (46,47). On the other hand, tumor cells in which p53 gene was lost or mutated not only withstood the deleterious effects of endogenous NO, but also exhibited faster growth and vascularity when transplanted *in vivo* (47). Since p53 mutation occurs in nearly half of human cancers (48), it was hypothesized that NO would facilitate tumor progression in a large proportion of well-established human tumors (47). We hypothesize that during the clonal evolution of tumors *in vivo*, high NO producing clones susceptible to NO-mediated injury are deleted and selected against those which are genetically or biochemically resistant to NO-mediated injury and capable of utilizing NO to their advantage for expression of an aggressive phenotype (25). Loss of functional p53 gene may represent one of many genetic changes which can possibly result in the above phenotype. Other mechanisms which have been shown to impart NO-resistance to a cell are upregulation of heat shock protein (HSP)70 (49) or cycloxyoxygenase (COX)-2 enzyme (50). That NO-resistance of tumor cell can lead to NO-dependence is suggested by the findings that iNOS knockout mice promote the growth of NO-sensitive tumour cells but retard the growth of NO-resistant tumor cells (51).

For further details on the role of NO in carcinogenesis and tumor progression, we direct the reader to three articles in which we have reviewed the subject including our own studies funded by this grant (25, 52, 53) (appendixes A-2, A-7, A-8).

C3H/HeJ mammary tumor model employed in the present project.

Details of this model are provided in appendix A-6. In brief, this model is a combination of spontaneous C3H/HeJ mammary tumors and some of their clonal derivatives produced in our laboratory. Approximately 90% of retired breeder females of this mouse strain spontaneously develop invasive mammary adenocarcinomas with a pseudoglandular architecture, all of which

metastasis to the lungs. Tumor development is due to insertional mutagenesis of certain cell growth-regulating loci resulting from the integration of the proviral form of the mouse mammary tumor virus (MMTV) in the developing mammary tissue of mice receiving the virus via mother's milk. Approximately 39% of human breast cancer specimens express a 660 bp sequence of the MMTV envelop gene (54), the epidemiological significance of which remain to be identified. This finding and the similarity in histological features and metastatic behaviour, as reported by us earlier (55), suggest that C3H/HeJ spontaneous mammary tumors may represent the closest model for the human breast cancer, in particular, the familial form. We have derived two clonal lines, C3L5 and C10, grown from a spontaneous mammary tumor-derived line T58. The metastatic phenotype for C3L5 is high, for C10 is low, and for T58 is intermediate, based on the number of spontaneous lung metastases from subcutaneously transplanted tumors.

Preliminary data provided in the original grant application and substantiated further in the last four annual reports revealed that spontaneous C3H/HeJ primary tumors expressed eNOS protein (based on immunocytochemistry) in a heterogeneous manner in tumor cells, whereas their metastases in the lungs were uniformly and strongly positive for eNOS (25). This finding suggested that eNOS bearing cells in the primary tumor were more prone to metastasis. This suggestion was strengthened by the findings that C3L5 cells (highly metastatic) were strongly positive for eNOS *in vitro*, as well as *in vivo* both at primary and metastatic sites (25). In addition, iNOS was inducible in C3L5 cells when cultured with IFN-γ and LPS (25). In contrast, C10 cells (poorly metastatic) were weakly positive for eNOS, and the expression was heterogeneous. These findings, combined with our observations (25,40,41) that two NOS inhibitors NMMA and L-NAME reduced the growth of C3L5 primary tumors as well as their spontaneous lung metastases, led us to hypothesize that tumor-derived NO promoted tumor progression in this mammary tumor model. A large component of the current project was to validate this hypothesis and to identify the mechanisms underlying NO-mediated promotion of tumor progression in this model.

Role of NO in "capillary leak syndrome"

We discovered that capillary leak syndrome (characterized by fluid leakage from the capillaries into tissue spaces, various organs and body cavities), a life-threatening side effect of interleukin-2 (IL-2) based cancer immunotherapy, is due to the increased production of nitric oxide (40,41,56,57). This was shown by (a) a positive correlation of NO levels in the serum and the body fluids with the severity of IL-2 therapy-induced capillary leakage in healthy and tumor-bearing mice, and (b) an amelioration of this capillary leakage by chronic oral administration of NOS inhibitors NMMA and L-NAME (see ref. 58 for a comprehensive review, provided as appendix A-3.

Unexpectedly, we also observed that additional therapy with NOS inhibitors improved antitumor/antimetastatic effects of IL-2 therapy (40,41). This finding led to the suggestion that NO induction by IL-2 therapy interfered with antitumor effects of IL-2 therapy. We tested this hypothesis by investigating the effects of addition of L-NAME on IL-2 induced generation of

lymphokine activated killer (LAK) cells *in vivo* and *in vitro* in healthy and tumor bearing mice (59). Results revealed that inhibition of NO production *in vivo* or *in vitro* by addition of L-NAME to IL-2 therapy or IL-2 induced lymphocyte activation *in vitro* caused a substantial enhancement of LAK cell activation. In other words, IL-2 induced NO production interfered with optional LAK cell activation which can be abrogated with NOS inhibitors (59).

A minor component of the current project was to (a) identify the cellular source of NO induced by IL-2 therapy, (b) identify the nature of structural damage to the lungs of mice suffering from IL-2 induced pulmonary edema and pleural effusion, and (c) examine the effects of L-NAME therapy on the above parameters. Results of these studies have been published (57) and one provided as appendix A-1. In brief, IL-2 therapy led to high levels of iNOS protein expression and activity in the tissues of the anterior thoracic wall in accompaniment with pleural effusion. There was structural damage to the lungs (alveolar epithelium and interstitial tissue) and its capillaries by IL-2 therapy, which were mitigated by L-NAME therapy. L-NAME therapy abrogated IL-2 induced rise in iNOS activity but not the expression iNOS protein in the tissues

6. BODY OF THE PROGRESS REPORT

Overall Hypothesis: Tumor derived NO promotes C3H/HeJ mammary tumor progression and metastasis.

Overall Objectives:

(1) To validate the hypothesis of the stimulatory role of NO in mammary tumor progression by further correlation of eNOS expression in clonally derived cell lines with tumor growth, metastasis, and angiogenesis *in vivo*, and migratory and invasive functions *in vitro*, and investigating the effects of blocking NOS activity or down-regulating eNOS gene on tumor cell behaviour *in vitro*, e.g. migration and invasiveness, and *in vivo*, e.g. tumor growth, angiogenesis and metastases.

(2) To identify mechanisms of NO-mediated stimulation of tumor progression by investigating the role of NO in tumor cell proliferation, migration, invasiveness and tumor-induced angiogenesis.

Our assessment of overall progress in relation to the statement of objectives

Task 1 Relationship between NOS expression and tumor progression/ metastasis:

Progress has matched with our expectations in components 1a and 1b. The molecular biology components have been frustrating. This was initially because of our failure to knockout the eNOS gene in C3L5 cells, evidently because of increased number (3.6) of gene copies in these cells. Subsequently, we adopted the antisense RNA approach to downregulate eNOS. We succeeded in obtaining low or nonexpressing clones more than once, however, all of them proved

to be unstable and reverted to the expresser phenotype. During the third and the forth year we applied antisense oligonucleotides to achieve downregulation of a shorter duration. While this was achieved with a few ethoxy-methoxy modified antisense oligos, the results were too transient for proceeding with the functional studies 1d and 1e. Consequently we abandoned this unproductive route of gene manipulation and relied more on the effective approach of enzyme inhibition, and use of tumor cell clones which naturally varied in their eNOS-expression.

Task 2 Identification of mechanisms promoting tumour progression by NO.

Although this task was initially assigned to Year II onwards, significant progress was achieved in year I and all the goals have been completed in Years II - IV. Based on our findings, we proposed some new experiments which have now been completed (see later).

Task 3 Mechanisms underlying IL-2 induced capillary leakage and interference with antitumour effects of IL-2 therapy by IL-2-induced NO.

Although this task was initially assigned to Year III onwards, we have completed our goals in this area ahead of the target date, leading to some newly proposed experiments. These experiments have now been completed (see later).

Record of Research findings during the past year, including the summary of findings in the previous years.

Task 1 Relationship between NOS expression and tumor progression and metastasis.

(a) Relationship between the expression of NOS protein and tumor growth and metastasis.

(i) Spontaneous C3H/HeJ mammary tumors.

We have completed the long process of accrual of a large number of spontaneous tumors developing during the life time of female retired breeder C3H/HeJ mice. During the project period we harvested a total of 41 spontaneous tumours and their metastatic foci for eNOS and iNOS immunostaining. The data confirm the findings presented in appendix A-6 (ref 60), which were based on 20 tumors (excluding 6 which were highly necrotic and thus not usable for immunostaining).

In summary, spontaneous tumors at the primary sites showed heterogeneous eNOS expression in tumour cells. Irrespective of tumor growth rates (whether fast, intermediate or slow) a mixture of strongly eNOS positive (40-70%) or completely eNOS negative cells (30-60%) (appendix A-6, Figure 2A) were observed, however, the proportion of positive cells were higher in poorly differentiated areas than in differentiated areas of tumors showing pseudoacinar arrangement of tumor cells (appendix A-6, Figures 2A, 2B). In contrast, virtually all tumor cells at the sites of lung metastasis were strongly and homogeneously eNOS positive (appendix A-6,

Figure 2C). iNOS expression was restricted to a subset of macrophages within the primary tumors or the tumor stroma (appendix A-6, Figure 2D) as well as the metastatic sites. These results provide a strong validation of our preliminary data (25) suggesting a positive association between eNOS expression and tumor growth and metastasis, leading to the hypothesis that eNOS expression provides tumor cells with a selective advantage for growth and metastasis.

(ii) C3L5 (highly metastatic) and C10 (weakly metastatic) cell lines and their transplants.

(a) The data are detailed in appendix A-6 (Ref 60)

In summary, subcutaneous transplants of C3L5 cells grew faster at the primary sites and produced a larger number of spontaneous lung metastasis than those of C10 cells during the same time span (Figure 1, appendix A-6). Weakly metastatic C10 cells expressed low levels of eNOS *in vitro* in a minor proportion of cells, as compared to the highly metastatic C3L5 cells which expressed high eNOS levels in nearly every cell. Similar differences in eNOS protein expression were observed *in vivo* only at the primary tumor site (Figures 2E and G, appendix A-6), however their lung metastasis were equally and strongly positive for eNOS (Figures 2F and 2H, appendix A-6). iNOS expression was undetectable in either cell line, but noted in a subset of both cell lines *in vitro* only when cultured in the presence of IFN-γ + LPS. C3L5 and C10 tumor cells grown *in vivo* were iNOS negative both at primary and metastatic sites; only a subset of macrophages at either site expressed iNOS. These results were objectively validated by quantitative image analysis (Figures 3A and 3B, appendix A-6), and supported the hypothesis of a causal relationship of eNOS expression to tumor growth and metastasis.

(b) Attempts to investigate biological alterations of murine mammary adeno-carcinoma cell line (C3L5) by downregulation of eNOS gene expression.

As reported earlier, we failed to knock out eNOS gene in the high eNOS expressing C3L5 cells because of the presence of a high copy number of eNOS genes (average 3.6). Subsequently, our attempts to knock down this gene by stable transfection with antisense RNA were also met with frustrations, because of the instability of all the eNOS down-regulated clones reverting to eNOS expressing phenotype, possibly because of a variety of reasons suggested or reported by others (61-63). Later on, we tried two more antisense constructs based on two different fragments of the eNOS gene (one derived from the tail region) to transform C3L5 cells at different concentrations using the lipofectamine method. These attempts were also unsuccessful.

During the last two years, we devoted a good deal of time and energy to apply antisense oligonucleotides knock down strategy to downregulated eNOS in C3L5 cells. In essence, these results were also disappointing, because the down-regulation was (a) not always consistent, and (b) not stable enough for the duration required for our functional assays. Many researchers believe that phosphothioate (PS) oligodeoxy-nucleotides inhibit protein expression by blocking the ribosome as it moves along mRNA. That is why the dogma is that the design of

oligonucleotides in the vicinity of AUG (start codon) site on the mRNA are most active. But because almost all PS oligos function as antisense molecules by forming duplex with target mRNA and then serves as substrate for RNase H, Dr. Nic Dean of ISIS Pharmaceutical recommends to design several oligo sequences throughout the mRNA (64). In fact, his group has shown in many instances (64-66) that PS-oligos designed in the vicinity of AUG (start codon) site have very little or no potential to inhibit target protein expressions, whereas the maximal ability to inhibit target protein expression was shown by PS-oligos designed from some other regions of the mRNA. Furthermore, the phosphothioate modifications of the oligodeoxy-nucleotides, although effective, have some limitations. Their main limitation is that they are metabolised in cells over time, leading to almost total loss of activity over 48-72 h period. Dr. Nic Dean's group has shown that the 2'-O-(2-methoxy) ethyl (2'-MOE) modification of oligodeoxynucleotide result in dramatic enhancement in the ability of the sequence to hybridize to a target mRNA and nuclease resistance when compared with PS-oligos (67). Therefore, we requested him to synthesise 2'-MOE modified antisense murine eNOS oligodeoxynucleotides for us. He kindly synthesized 22 different oligos designed from different regions of the eNOS mRNA for us, the sequences of which remain blinded to us. We utilized these oligos in C3L5 cell cultures in order to screen active sequences. Lipofectin was used as oligo uptake enhance for 4hr incubation, after which it was removed and replaced with media. The cells were then allowed to incubate overnight, and NO (nitrite and nitrate) concentrations in the medium were determined as the end result of eNOS mRNA down regulation.

The results of some six screening attempts were highly variable. In two attempts, three oligos significantly downregulated ($\geq$ 50%) NO production by C3L5 cells as compared with that noted with mock-treated controls. However, the effects were either too transient (i.e. undetectable after 72 hours) or not adequately reproducible in subsequent screening (data not presented). We were advised that our problems, may again, lie in the genetic constitution of our target cell line expressing a high copy number of the eNOS gene.

Last year we have decided that further attempts to downregulate eNOS gene in C3L5 cells are not worth pursuing for the following reasons: (a) we have achieved all the expected results by blocking NOS activity in these cells, and (b) we have, in essence, achieved similar objectives by conducting functional assays with our naturally occurring low eNOS-expressing C10 cell line clonally derived from the same parental tumor, from which the high eNOS-expressing C3L5 cell line was derived.

Task 2 Identification of mechanisms underlying NO-mediated promotion of tumor progression.

We hypothesised that tumour-derived NO facilitates tumor progression and metastasis by **(a)** promoting tumour cell migratory ability, **(b)** promoting tumor cell invasive ability and **(c)** promoting tumor-induced angiogenesis which is critical for the growth of solid tumors. We had already shown that tumor-derived NO exerted no influence on tumour cell proliferation *in vitro*. Others have shown that tumor-derived NO

promotes tumour blood flow and microcirculation which can indirectly promote tumor growth (68-70).

(a) Effects of tumor-derived NO on *in vitro* migratory ability of C10 and C3L5 tumor cells. (detailed in Appendices A-6, A-8, A-9; Ref 60, 53, 71)

Since migratory ability is an essential component of cellular invasiveness and metastasis, we examined the role of NO on the migration of the two mammary tumour cell lines differing in eNOS expression and metastatic phenotype. First, we compared the migration kinetics of the two cell lines using a transwell migration assay detailed in appendix A-6. Second, we examined the migratory ability of each cell line after treatment with the NOS inhibitor L-NAME in the presence or absence of excess L-arginine (the natural substrate for NOS, which should compete with L-NAME) under conditions detailed in appendix A-6, in order to identify the contributory role of NO which was measured in the medium under identical conditions.

Results (detailed in appendix A-6) revealed that the two cell lines did not differ significantly in their migratory abilities (Figure 4A, appendix A-6), however, migration of both cell lines were inhibited in the presence of L-NAME in a dose-dependent manner and restored in the additional presence of excess L-arginine (Figure 5A, appendix A-6). These treatments produced correspondingly similar effects on the NO production by these cells (Figure 6, appendix A-6). These results demonstrate that migration of both cell lines are stimulated by endogenous NO, in spite of differences in the level of NO production by these cells. Thus an absence of any significant difference in the basal migration rates of the two cell lines is possibly explained by differential production of other migration-regulating molecule(s) by these cells. The above is the first demonstration of migration stimulation of tumor cells by tumor-derived NO. The underlying mechanisms of signal transduction were then investigated with additional experiments, not proposed earlier.

We wished to identify intracellular pathways of signal transduction responsible for NO-mediated stimulation of tumor cell migration, utilizing the high eNOS-expressing C3L5 mammary tumor cell line. It has been shown that most physiological functions of NO (e.g. vasorelaxation by endothelium derived NO) are mediated by stimulation of guanylate cyclase leading to an elevation of intracellular cyclic GMP (cGMP) (10-12). In addition, vascular endothelial growth factor (VEGF)-mediated angiogenesis, involving endothelial cell proliferation, migration and tube formation has been shown to be dependent on stimulation of endothelial NOS (i.e. NO production) followed by activation of mitogen activated protein (MAP) kinase pathway (72,73). We postulated that endogenous or exogenous NO stimulate guanylate cyclase which causes an increase in the level of cGMP leading to the activation of cGMP dependent protein kinase (G kinase), MAP kinase kinase (MAPKK) and MAP kinase (extra cellular regulated kinases or ERK 1 and 2), followed by the activation of the myosin light chain kinase (MLCK) which, in turn, activate the cellular motility apparatus. Experiments were conducted with C3L5 cells to test this hypothesis: Detailed results are presented in appendix A-9 (Ref 71) and abstracted in appendix A-8 (Ref53) as well as numerous conference presentations (B-8, B-9, B-

10). They are summarized below.

We first confirmed that endogenous NO (due to eNOS expression) promoted C3L5 mammy tumour cell migration. This was shown by migration inhibition of the cells in vitro in the presence of the NOS inhibitor L-NAME in a concentration-dependent manner, and an abrogation of the inhibition (and on occasions, stimulation above basal levels) in the additional presence of excess L-arginine, the natural substrate of NOS. However, the presence of ODQ, a selective inhibitor of guanylate cyclase (73), not only blocked basal C3L5 cell migration, but also blocked the migration-restoring effects of L-arginine in L-NAME treated cells. This finding revealed that (a) migration of C3L5 cells is dependent on the activation of guanylate cyclase, i.e. cGMP-dependent, and (b) endogenous NO-mediated C3L5 cell migration is also cGMP dependent. The latter conclusion was further supported by the fact that addition of 8-Bromo cGMP, a cGMP analogue which can readily enter the cells, significantly stimulated migration of C3L5 cells, indicating that an elevation of cGMP level was migration stimulatory.

To test the requirement of the MAPK pathway in C3L5 cell migration, cells were treated with PD098059, a drug which selectively inhibits MAP Kinase Kinase (MAPKK or MEK) (73). This treatment not only blocked basal migration of C3L5 cells, but also blocked migration restoring effects of L-argininine in L-NAME treated cells. These results revealed that (a) C3L5 cell migration was dependant on MEK; and (b) endogenous NO-mediated migration stimulation in C3L5 cells required MEK activity.

We then tested whether endogenous or exogenous NO stimulated phosphorylation (activation) of two important members of the MAPK family ERK I and ERK II, as tested by western immunoblot analysis. Treatment of cells with NOS inhibitor L-NAME resulted in a time-dependent reduction in ERK I and ERK II phosphorylation. Addition of excess L-Arginine (to restore endogenous NO production) and sodium nitroprusside (SNP, an NO-donor) to L-NAME treated cells rapidly stimulated ERK I and ERK II phosphorylation above control levels. These results reveals that both endogenous and exogenous NO activated ERK I and ERK II in C3L5 cells.

Next, we tested whether NO-mediated activation of guanylate cylase and MAPK are linked. This was tested by measuring the levels of ERK I and ERK II phosphorylation in C3L5 cells treated with ODQ (to block guanylate cyclse and thus reduce cGMP levels) or 8 Bromo-cGMP (to increase cGMP levels). ODQ reduced and 8 Bromo-cGMP increased the levels of phosphorylation, indicating that MAPK stimulation in C3L5 cells was cGMP dependant.

Finally we tested whether NO-mediated stimulation of ERK phosphorylation was cGMP-dependent. The presence of guanylate cyclase inhibitor ODQ blocked the ERK phosphorylation-restoring effects of excess L-Arginine in L-NAME-treated cells, providing a link between NO metabolic, guanylate cyclase and MAPK pathways in mediating migratory responses in C3L5 cells. This is the first report of identification of these signaling events in NO-mediated migration-stimulation in a cancer cell.

(b) Effects of tumour-derived NO on invasiveness of mammary tumor cells.

(i). A comparison of *in vitro* invasiveness of highly metastatic (and high eNOS expresser) C3L5 with weakly metastatic (and low eNOS expresser) C10 cell lines: role of NO.

Materials, methods and results are detailed in appendix A-6 (Ref 60). In brief, C3L5 cells invaded matrigel at a faster rate than C10 cells (Figure 4B, appendix A-6). Furthermore, L-NAME caused a dose-dependent inhibition of invasion in both cells, which was relieved in the presence of excess L-arginine (Figure 5B, appendix A-6) with concomitant restoration of NO production (Figure 6, appendix A-6), indicating that endogenous NO stimulated invasiveness of both cell lines.

(ii). Mechanism of NO-mediated stimulation of invasiveness, investigated with C3L5 cells.

As presented in the 1997-1998 Progress Report, C3L5 cell invasiveness was shown to be dependent on endogenous NO.

Endogenous (eNOS-derived) NO was shown to downregulate TIMP-2 and TIMP-3 mRNA expression. In addition, when iNOS was induced in C3L5 cells by LPS+IFN-γ *in vitro*, these cells became more invasive, and there was an additional upregulation of MMP-2. Thus NO-mediated promotion of invasiveness was due to an altered balance between MMP-2 and its inhibitors TIMPs 2 and 3 (for details, see published ref. 74; appendix A-4).

(iii). Role of uPA-uPAR system in NO-mediated stimulation of tumour cell migration or invasiveness.

Urokinase type plasminogen activator (uPA) is a major protease made by cancer cells including the present mammary tumor model. uPA has the dual ability of stimulating migration as well as invasiveness of certain cancer cells. In the former case, the effect is independent of the uPA catalytic domain, resulting from binding of the aminoterminal domain of uPA to uPA-receptor (uPAR) which can mediate a migration-stimulatory signal. In the latter case uPAR bound uPA, via the catalytic domain exerts proteolytic action to activate MMP's which, in turn, can degrade the extracellular matrix.

To test whether uPA-uPAR system is involved in regulating C3L5 cell migration or invasiveness, we conducted migration and invasion assays in the presence of anti-uPA as well as anti-uPAR antibodies. As presented in 1999-2000 progress report, both antibodies significantly reduced C3L5 cell migration as well as invasiveness. We shall test whether NO stimulates the uPA-uPAR systems as follows: (1) We shall measure the uPA protease activity with casein zymography in the presence of NOS inhibitor L-NAME and NO donor SNP. (2) We shall investigate whether these treatments alter the expression of uPA and

uPAR mRNA with Northern blot analysis and uPAR expression with flow cytometry. These newly proposed experiments (which were not in the original proposal) will be completed with other funds.

(c) Effects of tumor-derived NO on tumor-induced angiogenesis.

(i) C3L5 tumour model

We have devised a novel tumor angiogenesis assay employing implants of **growth factor-reduced** matrigel, inclusive of tumor cells in the matrigel suspension (detailed in appendix A-5, Ref 75). Matrigel implants alone had no angiogenic effect, whereas inclusion of tumor cells caused significant angiogenesis. The time course, geography and levels of angiogenesis were objectively measurable after Masson's trichrome staining and CD31 (endothelial cell marker) immunostaining of sections. Animals received L-NAME (NOS inhibitor) or D-NAME (inactive enantiomer of L-NAME used as controls). D-NAME was found to have no effect on basal angiogenesis. Both drugs were given continuously via osmotic mini-pumps, to evaluate the effects of NOS inhibition on tumor-induced angiogenesis measured at 14 days after implantation of matrigel suspended tumor cells. Histological evaluation of implants revealed that neovascularization initially started in the periphery of the implants with concurrent development of stroma. Developing tumors were then fed by secondary vessels growing from the stromal region. At later time points (e.g. 14 days), necrosis was evident in the deeper areas of tumors. It was found that L-NAME treatment caused a dramatic inhibition of angiogenesis both in the stroma as well as tumor tissue as compared to D-NAME, and also a relative reduction in viable tissue mass (stroma and tumor) and an increase in necrosis. Antiangiogenic effects of L-NAME therapy were evident from day 12 onwards. Detailed results are presented in appendix A-5. These data show that NO is a key mediator of C3L5 tumor-induced angiogenesis, and that growth inhibitory effects of L-NAME on the primary tumor are partly mediated by reduced tumor-angiogenesis.

(ii) A comparison of C3L5 (high eNOS expresser) and C10 (low eNOS expresser) tumour models

Materials, methods and results are detailed in appendix A-6 (Ref. 60). In brief, in the matrigel implant assay, C3L5 cells were more angiogenic than C10 cells, as expected from their differences in eNOS expression (Figure 7, appendix A-6). Unexpectedly, however, L-NAME treatment did not significantly affect C10 tumor induced angiogenesis indicating that either a certain threshold of NO level was required for angiogenesis stimulation, or that a differential upregulation of other angiogenic factor(s) may have compensated for the L-NAME inhibition of angiogenesis in C10 cells.

We have discovered that C3L5 cells express VEGF, a potent angiogenic factor. VEGF-induced angiogenesis (endothelial cell proliferation, migration and tube formation) has recently been shown to be dependent on NO production in endothelial cells following eNOS activation

(evidently due to increase in intracellular calcium), leading to stimulation cGMP and then activation of MAPK pathway (72,73). Thus, it is likely that VEGF expression by C3L5 cells is an additional tool for NO-mediated angiogenesis because of eNOS activation in tumor cells as well as endothelial cells. We shall test whether a neutralisation of VEGF activity (with a VEGF neutralizing antibody) in C3L5 cells *in vitro* causes a reduction in Ca++ dependent NO production by C3L5 cells in culture medium. These will be expected if VEGF activates eNOS in C3L5 cells. We shall also test whether this treatment causes a reduction in intracellular Ca++ level in C3L5 cells. These experiments will be completed with other funds.

Task 3 Role of NO in IL-2 induced capillary leakage and mechanisms by which this NO- production compromises antitumor effects of IL-2 therapy.

This task, as initially proposed, was completed earlier and published (Ref 57-59, Appendices A-1, A-3). We showed that IL-2 therapy induced active iNOS in tissues contiguous with pleural effusion and the resulting NO overproduction caused structural damage to the lungs and its capillaries. These injuries were ameliorated with the NOS inhibitor L-NAME.

These results raised the following questions. Was the damage to the lungs and its capillaries due to a direct injury (structural damage and apoptosis) by NO, or injury by certain reaction product of NO? Recently, it has been reported that oxygen-free-radicals play a role in IL-2 therapy-induced capillary damage because it could be ameliorated with dimethylthiourea, a scavenger of oxygen-free-radicals (76). We hypothesised that formation of peroxynitrite, a potent endotheliotoxic molecule, due to a combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary leakage.

Since cytotoxicity due to peroxynitrite is reported to be due to a nitration of important intracellular tyrosine kinases to form nitrotyrosine, nitrotyrosine is considered to provide a good marker for peroxynitrite-mediated cellular injury. We started testing these hypothesis by immunostaining tissues of IL-2-treated mice for nitrotyrosine with the expectation that this marker would appear strongly in tissues of IL-2-treated mice showing capillary leakage, and diminish in mice treated with IL-2 in combination with the NOS inhibitor L-NAME. In the 1998-99 annual report, we presented some preliminary data indicating that normal lungs stained for nitrotyrosine irrespective of IL-2 therapy, possibly because of high levels of basal NOS activity in the normal lungs. However, kidneys (in particular, medullar regions) provided some discrimination for nitrotyrosine as a marker for IL-2-induced capillary leakage.

In 1999-2000, we received a fresh batch of human recombinant IL-2 from Chiron Corporation and applied the following protocol to 8-10 week old C3H/HeJ mice (5-6 mice/group): (a) IL-2 injection alone, 50×10^3 Cetus units i.p. every 8 hours x 10 injections; (b) IL-2 therapy as in (a) combined with continuous therapy with L-NAME given subcutaneously with minipummps for the whole duration (0.5 ml/hr.; 25 mg/200 µl 0.9% NaCl); (c) IL-2 therapy in combination with D-NAME (inactive enantiomer of L-NAME) as above; (d) Therapy with vehicles alone (control). Animals were sacrificed shortly after the injection protocol to measure

(a) pleural effusion, (b) water content (wet/dry ratio) of the lungs, (c) water content (wet/dry ratio) of the kidneys as markers for capillary leakage. Frozen sections of kidneys were immunostained for nitrotyrosine. Sections immunostained with nitrotyrosine antibody preabsorbed with nitrotyrosine provided specificity controls. Capillary leakage data were analysed with one way analysis of variance.

Results presented in the 1999-2000 annual report showed that (1) IL-2 alone or IL-2 + D-NAME caused significant pleural effusion and capillary leakage in the lungs as well as the kidneys. There was no difference between these two groups. (2) Addition of L-NAME therapy significantly reduced the IL-2 induced pleural effusion and capillary leakage in the lungs and the kidneys, however, they were not completely ameliorated. Results on immunostaining for nitrotyrosine in the kidneys in the treated mice were consistent with our hypothesis: (a) Strong immunostaining for nitrotyrosine was detected in IL-2 or IL-2 + D-NAME treated mice in their kidneys. The staining was more prominent in the medulla than in the cortex. The staining were abolished by pre-absorption of the primary antibody with nitrotyrosine, indicative of staining specificity. No difference was found between IL-2 and IL-2+D-NAME treated groups. (b) L-NAME therapy, nearly completely abrogated this immunostaining indicating that abrogation of capillary leakage went hand-in-hand with nitrotyrosine staining. While these correlative data do not establish a cause and effect relationship between the production of peroxynitrite (as indicated by nitrotyrosine staining) and IL-2 induced capillary leakage, they are highly suggestive. In future experiments, we wish to test whether therapy with peroxynitrite scavengers, e.g. Lazaroids (77), can block or reduce IL-2 induced capillary leakage as well as nitrotryosine immunostaining in the tissues.

In a companion study (not proposed in the original project proposal) last year, we tested whether VAA, a lectin derived from the plant mistletoe, reported to have an immunostimulant role in vitro and vivo (due its reported ability to stimulate IL-2 receptors on lymphocytes or induce cytokine production in vivo) has any additional beneficial effect when combined with IL-2 therapy in our C3L5 mammary tumor model. We measured the effects of VAA therapy on its own or in combination with IL-2 therapy on tumor growth and metastasis, fluid retention in the pleural cavity, lungs and the kidneys, NO levels in vivo, and immunoreactive nitrotyrosine accumulation in kidneys (used as a marker of capillary leakage). The results are detailed in appendix A-10.

We unexpectedly found that therapy with VAA alone stimulated the growth of primary tumors as well as their spontaneous metastases in the lungs of mice transplanted with C3L5 mammary tumor cells. This was in spite of the fact that VAA did not influence NO producing ability of C3L5 tumor cells in vitro. It is likely that VAA stimulated tumor growth-inducing cytokine(s) in vivo. When combined with IL-2 therapy, VAA had NO beneficial or detrimental influence on the antitumor and antimetastatic effects of IL-2 therapy, IL-2 induced capillary leakage, or IL-2 induced NO production in vivo and nitrotyrosine accumulation in the kidneys. These results (detailed in appendix A-10) raises caution against VAA-based cancer therapy, which has recently gained some popularity (and notoriety) as an anticancer agent in some European countries amongst practitioners promoting "alternative" forms of cancer therapy.

7. KEY RESEARCH ACCOMPLISHMENTS

Following were the achievements during the project period.

I. We have expanded and validated our earlier data showing that:

(a) Spontaneous primary C3H/HeJ tumors show a heterogeneity in eNOS-bearing tumor cells; this expression was unrelated to tumor growth rate. However, the incidence of eNOS bearing cells was higher in undifferentiated than in differentiated zones of primary tumors, and metastatic foci resulting from each primary tumor was mostly eNOS positive.

(b) All C3L5 tumor cells (a highly metastatic clone of a spontaneous tumor) expressed eNOS *in vitro*; a minority expressed iNOS under inductive conditions (IFN-γ + LPS). When transplanted *in vivo,* most tumor cells at the primary site and a high proportion at the metastatic site expressed eNOS. C10 tumor cells originally shown to be a poorly metastatic clone of the same spontaneous tumor were shown to have a lower *in vivo* growth rate of primary tumors and a lower rate of spontaneous lung metastasis than C3L5 cells. These differences were positively correlated with their differences in eNOS protein expression *in vitro* as well as *in vivo* in primary tumors but not in metastatic foci which were equally positive for eNOS.

These findings substantiated further our hypothesis that eNOS expression provided an advantage for metastasis.

II. We abandoned our futile attempts to knockout eNOS gene in C3L5 cells because we found that they have increased (3.6) number of gene copies. Subsequently we adopted the alternative approach of downregulating eNOS by antisense RNA transfection and isolated eNOS downregulated clones. However, all the clones proved to be unstable and thus could not be applied to functional assays *in vitro* or *in vito*. Application of ethoxy-methoxy derivatives of antisense oligos for short-term biological assays, also proved to be non-productive and thus abandoned. However, the same objectives were achieved with inhibitors of NOS activity and use of a naturally occurring low eNOS-expressing clone.

III. **(a)** We have shown that endogenous NO promoted migratory function of C3L5 and C10 tumor cells.

This is the first definitive evidence of NO-mediated stimulation of tumor cell migration, which is an essential component of invasion and metastasis.

(b) We have identified the pathways for signal transduction for NO-mediated stimulation of tumor cell migration. We have shown that it involves stimulation of the guanylate cyclase followed by stimulation of the MAP kinase pathway.

This is the first demonstration of signal transduction pathways responsible for NO-mediated stimulation of migration of a cancer cell.

IV. **(a)** We have shown that endogenous NO promoted invasiveness of C3L5 and C10 tumor cells. The invasive function of the highly metastatic C3L5 cell line was investigated in detail. Their invasiveness was further stimulated by additional NO production when treated with IFN-γ and LPS because of the induction of iNOS in tumor cells.

This is the first definitive evidence of NO-mediated promotion of tumor cell invasiveness.

(b) We have identified some of the mechanisms responsible for NO-mediated stimulation of invasiveness. Endogenous and IFN-γ + LPS-induced NO down-regulated the expression of TIMP-2 and TIMP-3 genes. Induced NO further up-regulated the expression of MMP-2 gene. Thus, NO-mediated promotion of invasiveness resulted from an alteration in the balance between MMP-2 and TIMP's.

This is the first demonstration of mechanisms for NO-mediated promotion of tumor cell invasiveness.

V. By devising a novel tumor-induced angiogenesis assay *in vivo,* we have obtained definitive data showing that endogenous NO promotes C3L5 tumor-induced angiogenesis, which was higher than the level of angiogenesis induced by C10 cells expressing a lower level of eNOS.

This novel and objective angiogenesis assay is highly suitable for testing anti-angiogenic agents against human tumor cells grown in nude mice.

VI We had shown that active, inducible NOS expression, leading to high NO production *in vivo* is responsible for IL-2 therapy-induced capillary leakage in healthy mice. We identified the iNOS-expressing cells in the vicinity of the leakage (pulmonary edema, pleural effusion) and have shown that NOS inhibitors can restrain the IL-2 therapy-induced structural damage to the lungs. We have provided further evidence in support of our hypothesis that NO-mediated capillary damage following IL-2 therapy is owing to the formation of peroxynitrite, as revealed by immunostaining for nitrotyrosine in the kidneys, which was abrogated with L-NAME therapy. We have utilized this marker to evaluate capillary leakage in IL-2 based cancer immunotherapy, for example, in combination with VAA, an immunostimulatory plant-derived lectin. We showed that VAA therapy on its own or in combination with IL-2 provided no anticancer activity, nor did it influence IL-2 induced capillary leakage.

In summary, our progress matched with our expectations in most areas. In one

area we had no progress because of unexpected difficulties arising from the genetic constitution of our tumor cells. In other areas we had an accelerated progress, leading to some newer proposals and experimental data within the overall objectives of the project.

8. REPORTABLE OUTCOMES

A. Journal Publications (published, in press or submitted)

A1. Orucevic A, Hearn S, Lala PK: The role of active inducible nitric oxide synthase expression in the pathogenesis of capillary leak syndrome resulting from interleukin-2 therapy in mice. Lab Investigation. 76, 53-75, 1997.

A2. Lala PK, Orucevic A: Role of nitric oxide in tumor progression: Lessons from experimental tumors. Cancer and Metastasis Reviews. 17: 91-106, 1998.

A3. Orucevic A, Lala PK: Role of nitric oxide in interleukin-2 therapy induced capillary leak syndrome. Cancer and Metastasis Reviews. 17: 127-142, 1998.

A4. Orucevic A, Bechberger J, Green AM, Shapairo RA, Billiar TR and Lala PK: Nitric oxide production by murine mammary adenocarcinoma cells promotes tumor cell invasiveness. Int J Cancer 81: 889-896, 1999.

A5. Jadeski LC, Lala PK: NOS inhibition by N^{G}-Nitro-L-Arginine Methyl Ester (L-NAME) inhibits tumor-induced angiogenesis in mammary tumors. Amer J Path 155: 1381-1390, 1999.

A6. Jadeski LC, Hum KO, Chakraborty C, Lala PK: Nitric oxide promotes murine mammary tumor growth and metastasis by stimulating tumor cell migration, invasiveness and angiogenesis. Int J Cancer 86: 30-39, 2000.

A7. Lala PK and Chakraborty C: Role of Nitric Oxide in Carcinogenesis and Tumour Progression. Lancet Oncology 2(3) 149-156, 2001

A8. Jadeski LC, Chakraborty, C and Lala PK: Role of Nitric Oxide in Tumour Progression with Special Reference to a Murine Breast Cancer Model. Canadian J. Physicol, Pharmocal, (Proceedings of a symposium), accepted for publication.

A9. Jadeski LC, Chakraborty, C and Lala PK: Nitric Oxide mediated promotion of mammary tumour cell migration: Roles of guanylate cyclase and MAP Kinase pathways. Submitted for publication.

A10. Timoshenko, AV, Lan Y., Gabius HJ and Lala PK: Immunotherapy of C3H/HeJ

mammy adenocarcinomas with interlenkin-2, mistletoe lectin or their combination: effects on tumor growth, capillary leakage and nitric oxide (NO) production. Europe J Cancer, in press, 2001.

B. Conference presentations (Abstracts/extended abstracts)

B1. Lala PK, Hum K, Jadeski I, Orucevic A: Nitric Oxide (NO) mediated mammary tumor progression: Role of NO in tumor cell invasiveness. Proceedings of the Department of Defence Breast Cancer Program Meeting, Era of Hope, Vol. 2, 709-710, 1997.

B2. Hum K, Lala PK: Nitric oxide synthase expression promotes murine mammary tumor progression and metastasis. Proc Amer Assoc Cancer Res 39: # 1450, 212, 1998.

B3. Jadeski L, Lala PK: Role of nitric oxide in mammary tumor angiogenesis. Proc Amer Cancer Res 39, # 2574, 378, 1998.

B4. Hum K, Jadeski L, Lala PK: Nitric oxide synthases and murine mammary tumor progression. Proc Amer Assoc Cancer Res 40: # 3715, 563, 1999.

B5. Lala PK, Hum K, Jadeski L: Nitric oxide (NO) synthase expression promotes growth and metastasis of murine breast cancer cells due to invasion and migration stimulation by NO. Abstract: Reasons for Hope Conference in Breast Cancer Research, sponsored by the Canadian Breast Cancer Research Initiative. p 185, 1999.

B6. Jadeski L, Lala PK: The role of nitric oxide in murine mammary tumor induced angiogenesis. Abstract: Reasons for Hope Conference in Breast Cancer Research, sponsored by the Canadian Breast Cancer Research Initiative. p 182, 1999.

B7. Lala PK, Jadeski L, Hum K, Rozic J and Chakraborty C: Cyclooxygenase and nitric oxide synthase inhibitors inhibit murine mammary tumor growth and metastasis by blocking tumor cell migration, invasion and angiogenesis. Proc. AACR-NCI-EORTC International Conf. Abstract # 406, p 83, 1999.

B8. Jadeski L, Hum KO, Gleeson LM, Chakraborty C and Lala PK: Nitric oxide mediated promotion of murine mammary tumor cell progression: Role in tumor cell migration. Proc. Amer Assoc Cancer Res 41, Abstract # 1024, p 160, 2000.

B 9. Jadeski LC, Chakraborty C and Lala PK: Nitric Oxide-mediated promotion of mammary tumor cell migration is mediated by signaling through cyclic GMP and MAP kinase pathways. Proc. Amer. Assoc. Cancer Res. 42, Abstract #1619, p301, 2001.

B 10. Jadeski LC, Chakraborty C and Lala PK: Nitric Oxide promotes mammary tumor cell migration by activating cGMP and MAPK pathways. Abstract: Reasons for Hope

Conference, Canadian Breast Cancer Research Initiative. p76, 2001.

C. Degrees obtained that are supported by this award:

1. Amila Orucevic Ph D

"Mechanisms in IL-2 induced capillary leak syndrome"
The last component in her thesis was supported by this award. She completed her post-doctoral training with Dr. Tim Billiar (Pittsburg) and then completed her residency training in pathology (Rush Presbyterian St Luke's Medical Centre).

2. Kathleen Hum MSC

"Nitric Oxide Synthases and Murine Mammy tumor Progression"
She is currently a forensic scientist.

3. Lorraine Jadeski Ph D (Degree near completion)
"Mechanisms in Nitric Oxide-mediated murine mammary tumor progression"

D. Personnel serried by this award:

(a). Graduate students: Amila Orucevic (only a minor component of her study), Kathleen Hum, Lorraine Jadiski

(b). Research Technicians: Jenny Sun, Yan Lan

(c). Post-doctoral fellows: Wenling Lu, Alexander Timoshenko (partial support), primarily funded by an International Technology Transfer Fellowship Award of the International Union Agent Cancer.

(d). Research Associate: Chandan Chakraborty

(e). Research Assistants: Jerry Rozic

E. Research grants obtained / submitted on the basis of results obtained with this award:

(a). "Reciprocal Influence of Nitric Oxide and prostaglandins in Breast Cancer Progression" Three year operating grant awarded by the Canadian Breast Cancer Research Initiative (CBCR1) July, 2001 – June 2004 to Dr. PK Lala, P.I.

(b). Post-doctoral traineeship grant award (applied for) to the US Army Medical Research Materiel Command, BCRP, by Alexander Timoshenko under the suppression of Dr. P.K.

Lala.

9. CONCLUSIONS

Results of this project reveals that:

(a) Tumour-derived nitric oxide promotes murine mammary tumor progression by multiple mechanisms including stimulation of tumor cell migration, invasiveness and tumor-induced angiogenesis. Migration stimulation is mediated by activation of guanylate cyclase and MAP kinase pathways, and invasion stimulation is caused by a change in the balance of matrix metalloproteases and their inhibitors.

Since NOS activity correlates with the progression of human breast cancer, the above information is highly relevant for designing breast cancer therapy in the human. NOS inhibitors should have a valuable role by blocking multiple steps in breast cancer progression and metastasis.

(b) Induction of iNOS leading to increased NO production and peroxynitrite formation in various tissue is responsible for IL-2 induced "capillary leak syndrome" which can be mitigated with NOS inhibitors. NOS inhibitors also improved the anti-cancer effects of IL-2 therapy.

High-dose IL-2 therapy, in spite of proven benefit in certain human cancers, has lately been abandoned because of this side effect. This therapy can now be revived in combination with selective iNOS inhibitors.

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APPENDICES

A1. Orucevic A, Hearn S, Lala PK: The role of active inducible nitric oxide synthase expression in the pathogenesis of capillary leak syndrome resulting from interleukin-2 therapy in mice. Lab Investigation. 76, 53-75, 1997.

A2. Lala PK, Orucevic A: Role of nitric oxide in tumor progression: Lessons from experimental tumors. Cancer and Metastasis Reviews. 17: 91-106, 1998.

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The Role of Active Inducible Nitric Oxide Synthase Expression in the Pathogenesis of Capillary Leak Syndrome Resulting from Interleukin-2 Therapy in Mice

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SUMMARY: Previously, we showed that nitric oxide (NO) plays a major role in the pathogenesis of IL-2–induced capillary leak syndrome in healthy or mammary adenocarcinoma-bearing C3H/HeJ mice. NO synthase (NOS) inhibitors, such as N^G-nitro-L-arginine methyl ester (L-NAME) reduced all the manifestations of IL-2–induced capillary leakage, without compromising the antitumor effects of IL-2. The present study was carried out on healthy C3H/HeJ mice subjected to one or two 4-day rounds of systemic IL-2 therapy with or without oral L-NAME therapy to: (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) identify histologically the nature of the structural damage to the lungs associated with IL-2 therapy-induced pulmonary edema; and (c) evaluate the effects of additional L-NAME therapy on the above-mentioned parameters. Results revealed that IL-2 therapy in healthy mice resulted in the expression of inducible NOS in numerous tissues including the endothelium and muscles of the anterior thoracic wall as well as splenic macrophages. One round of IL-2 therapy resulted in high levels of inducible NOS (iNOS) activity in the anterior thoracic wall accompanied by pleural effusion. After two rounds of IL-2 therapy, there was neither pleural effusion nor high iNOS activity in the thoracic wall. IL-2–induced pulmonary edema after one round of therapy correlated to both a significant rise in NO production measured in the serum and structural damage to the lungs and its capillaries. Addition of the NOS inhibitor L-NAME totally eradicated NOS activity but not necessarily iNOS expression. It also reduced IL-2–induced pulmonary edema and pleural effusion, restrained the rise in the levels of NO metabolites (nitrites and nitrates) in the serum and pleural effusion, and significantly restored the structural integrity of the lungs after one round of therapy. Thus, NOS inhibitors may be beneficial adjuncts to IL-2 therapy for cancer and infectious diseases. (*Lab Invest 1997, 76:53–65*).

IL-2 therapy alone or in combination with ex vivo-generated lymphokine-activated killer (LAK) cells (Rosenberg, 1989; Fisher et al, 1988; Dutcher et al, 1989) or immunomodulators, such as indomethacin (Mertens et al, 1993a, 1993b), has shown some promising results in treating patients with certain forms of cancer, particularly melanomas and renal cell carcinomas. However, capillary leak syndrome (CLS), which is characterized by retention of extravascular fluid and severe hemodynamic instability inclusive of hypotension, remains a major roadblock to IL-2-based immunotherapy for cancer and infectious diseases (Siegel and Puri, 1991; Oppenheim and Lotze, 1994).

Excessive production of nitric oxide (NO), a short-lived biologic mediator (Palmer et al, 1987; Knowles and Moncada, 1994) synthesized—with the help of a family of enzymes called NO synthases (NOS)—by many mammalian cells from the amino acid L-arginine (Knowles and Moncada, 1994; Morris and Billiar, 1994), has been implicated in the pathogenesis of septic and endotoxic shock (Petros et al, 1991; Kilbourn et al, 1990; Wright et al, 1992). The high NO levels found in cancer patients receiving IL-2 therapy (Hibbs et al, 1992; Ochoa et al, 1992; Miles et al, 1994)

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seem to be responsible for severe hypotension, a common side effect of high-dose IL-2 therapy, because the condition is controllable with NOS inhibitors (Kilbourn et al, 1995; Fonseca et al, 1994).

We previously demonstrated (Orucevic and Lala, 1996c, 1996d) that moderate to high doses of IL-2 therapy in healthy or mammary adenocarcinoma-bearing C3H/HeJ mice resulted in capillary leakage, as indicated by pleural effusion and fluid retention in the lungs, spleen, and kidneys. We also showed that stable metabolic products of NO measured in the pleural effusion and the serum of these animals were directly related to the IL-2 dose. It was also shown that therapy with L-NAME, an NOS inhibitor administered in the animals' drinking water, significantly mitigated all of the manifestations of IL-2–induced CLS and led to a concomitant reduction of IL-2–induced NO production in the serum and pleural effusion. These results, combined with our previous findings of mitigation of IL-2–induced capillary leakage with oral N^G-methyl-L-arginine (NMMA), another NOS inhibitor (Orucevic and Lala, 1996a), strongly indicate that NO plays a major role in the pathogenesis of CLS induced by high doses of IL-2.

We hypothesize that IL-2 therapy directly or indirectly causes inducible NOS (iNOS) protein and iNOS activity in specific cells, causing an increase of NO production in local tissues, which leads, in turn, to CLS through the dual mechanism of NO-induced damage to endothelial cells (Palmer et al, 1992; Estrada et al, 1992) and vasodilation (Palmer et al, 1987). Whereas the former caused a direct leakage of the capillaries, the latter also resulted in systemic hypotension, which then indirectly caused pulmonary hypertension, thus further precipitating the pulmonary edema. Both mechanisms may also underlie the IL-2–induced pleural effusion in the mouse inasmuch as the blood in the murine pleura is partially derived from the pulmonary arteries (Pinchon et al, 1980). The present study was therefore designed in healthy C3H/HeJ mice to: (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) histologically identify the nature of structural damage to the lungs during IL-2 therapy-induced pulmonary edema; and (c) test whether the addition of L-NAME therapy abrogated the increase in NOS activity and IL-2–induced structural damage to the lungs. NOS activity of the lungs and the anterior thoracic wall, iNOS expression in the lungs, anterior thoracic wall, and spleen, as well as morphologic changes in the lungs were all evaluated in mice subjected to systemic IL-2 therapy with or without oral L-NAME therapy.

Results

Effects of L-NAME on IL-2–Induced Pleural Effusion and $NO_2^- + NO_3^-$ Levels in the Serum and Pleural Effusion after the First Round of Therapy

As we have previously reported in other studies (Orucevic and Lala, 1996d), L-NAME significantly reduced pleural effusion induced by IL-2 after the first round of therapy (Fig. 1, top). We also confirmed that IL-2–induced increases in $NO_2^- + NO_3^-$ levels in the pleural effusion are significantly reduced by addition of L-NAME (Fig. 1, bottom), a finding similar to those reported in our earlier studies (Orucevic and Lala, 1996d).

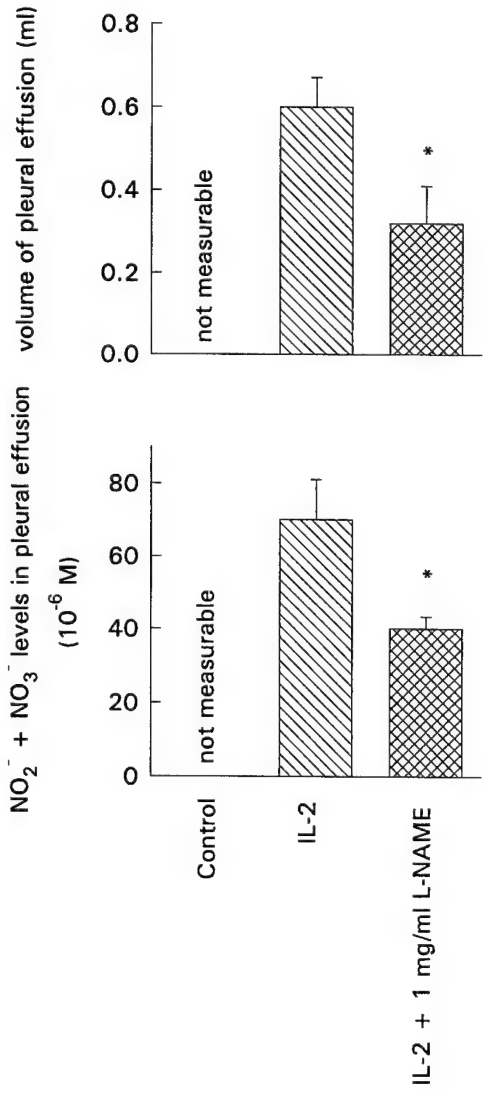

Figure 1.
Pleural effusion (top) and $NO_2^- + NO_3^-$ levels in the pleural effusion (bottom) after IL-2 and IL-2 + L-NAME therapy. Data represent mean ± SE ($n = 5$); *indicates significant difference from IL-2 treatment ($p < 0.05$). L-NAME therapy (1 mg/ml of drinking water) significantly ($p < 0.05$) reduced IL-2 (15,000 U/inj) induced pleural effusion and IL-2–induced rise in nitrite and nitrate levels in the pleural effusion. Control (untreated) mice did not show any pleural effusion.

Effects of L-NAME on IL-2–Induced Morphologic Changes of the Lungs after the First Round of Therapy

Light microscopic image analysis (Mocha Image Software; Jandel Scientific, San Rafael, California) of semithin sections of the lungs (Fig. 3) revealed that, in comparison with the control lungs (Fig. 3 and Table 1), IL-2 therapy led to a significant increase in the area occupied by the connective tissue in the lung and, conversely, a significant reduction in the area occupied by air spaces as a result of IL-2–induced pulmonary edema (Fig. 2). The addition of L-NAME therapy, however, significantly reduced IL-2–induced pulmonary edema (Fig. 2) and significantly restored the balance between the area occupied by the connective tissue and air spaces to close to control levels (Fig. 3 and Table 1).

Ultrastructural analysis of the lungs (Fig. 4) revealed that IL-2 therapy led to major distortions in the capillary ultrastructure of the lungs. The changes included swelling of endothelial cells and type I pneumocytes, thickening of the basement membrane of the thin portion of the capillaries, or herniation of the endothelial cell into the vessel lumen because of accumulation of fluid between the plasma membrane and the basement membrane, and the presence of cellular debris in the blebs in endothelial cells or type I pneumocytes, indicating cellular damage (Fig. 4). Addition of L-NAME therapy reduced the ultrastructural damage induced by IL-2. Although some swelling of endothelial cells remained, there was no noticeable damage of endothelial cell membranes or thickening of the basement membrane of the thin portion of the capillaries (Fig. 4).

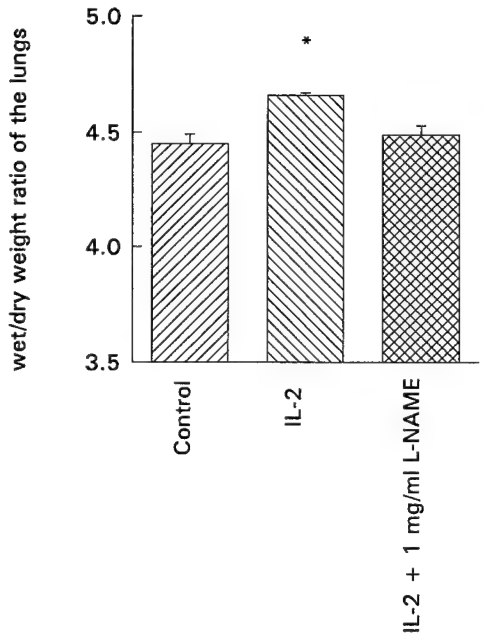

Figure 2.
Water content in the lungs after one round of IL-2 and IL-2 + L-NAME therapy. Data represent mean ± SE ($n = 5$). *indicates significant difference from control ($p < 0.05$). Addition of L-NAME (1 mg/ml of drinking water) significantly ($p < 0.05$) reduced IL-2 (15,000 U/inj) induced pulmonary edema.

Table 1. Percentage of Area Occupied by the Connective Tissue in the Lungs[a] after IL-2 and L-NAME therapy, expressed as mean ± SE ($n = 5$).

Treatment	% of Connective Tissue Area
IL-2	69 ± 2.5[b]
IL-2 + L-NAME	62.4 ± 1.3[c]
Control	55.2 ± 3.1

[a] Light microscopic image analysis (Jandel Scientific Mocha image software) of semithin sections of the lungs from animals treated with IL-2 (15,000 CU/inj., 10 inj., i.p. every 8 hours) or IL-2 + L-NAME (1 mg/ml of drinking water, starting 1 day before IL-2 therapy); [b] significant ($p < 0.05$) increase in the relative area occupied by the connective tissue, compared to control; [c] significant ($p < 0.05$) reduction in the relative area occupied by the connective tissue, compared to IL-2.

Effects of L-NAME on IL-2–Induced Capillary Leakage and NO Production after Two Rounds of Therapy

IL-2 therapy caused an increase in the water content in the lungs after the second round of therapy, however, this was not significantly affected by the addition of L-NAME therapy (Fig. 5). Similarly, L-NAME therapy at this point was not effective in causing a significant reduction in the $NO_2^- + NO_3^-$ levels in the serum (data not presented). There was no pleural effusion at this time in animals treated with either IL-2 alone or IL-2 with L-NAME, as had been documented earlier (Orucevic and Lala, 1996c, 1996d).

NOS Activity in the Lungs and Anterior Thoracic Wall after One or Two Rounds of IL-2 and L-NAME Therapy

One round of IL-2 therapy significantly increased Ca^{2+}-independent (primarily explained by inducible) NOS activity in the anterior thoracic wall (Fig. 6), whereas the increase in Ca^{2+}-independent NOS activity in the lungs was not significant (Fig. 7). The second round of IL-2 therapy, however, increased Ca^{2+}-independent NOS activity in the lungs but did not induce any Ca^{2+}-independent NOS activity in the thoracic wall (Figs. 6 and 7). The addition of L-NAME therapy during the first or the second round of IL-2 therapy eradicated NOS activity in both the lungs and anterior thoracic wall (Figs. 6 and 7).

Tissue Distribution of Immunoreactive iNOS Protein During IL-2 with or without L-NAME Therapy

Thoracic Wall. Immunocytochemical staining for iNOS enzyme revealed that one round of IL-2 therapy

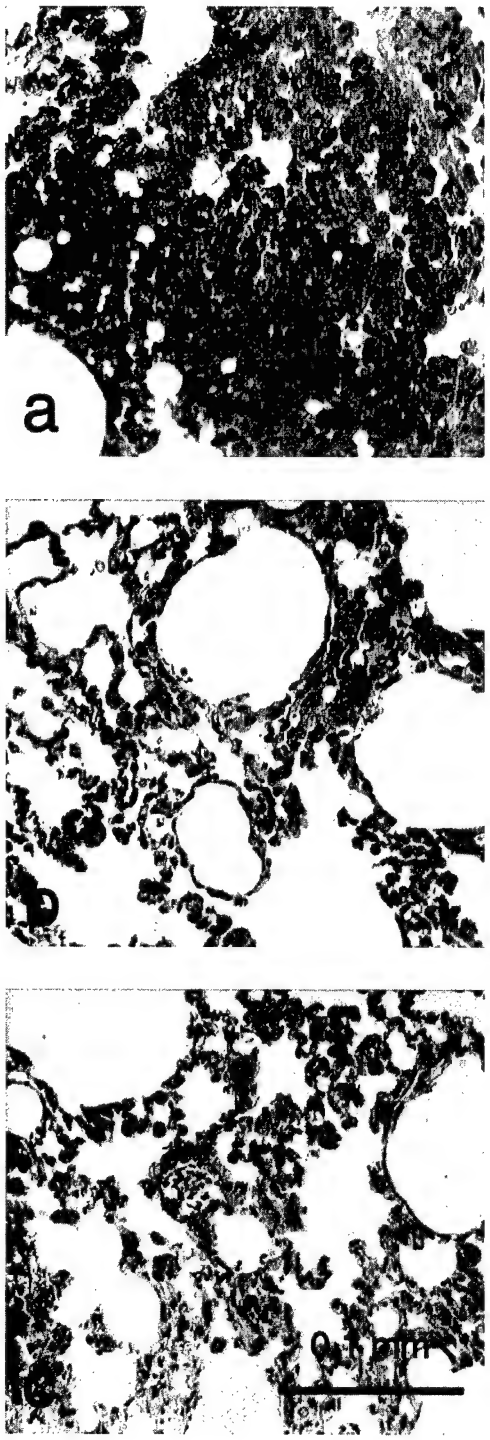

Figure 3.
Effects of IL-2 ± L-NAME therapy on histology of the lungs. a, IL-2; b, IL-2 + L-NAME; c, control; semithin sections of the lungs stained with toluidine blue. Sections of the lungs from IL-2–treated animals showed a significant increase in the area occupied by the connective tissue of the lungs as well as interstitial mononuclear cell infiltration (a). The addition of L-NAME therapy significantly reduced IL-2–induced pulmonary edema and mononuclear cell infiltration and restored the balance between the area occupied by the connective tissue and air spaces (IL-2 + L-NAME versus control).

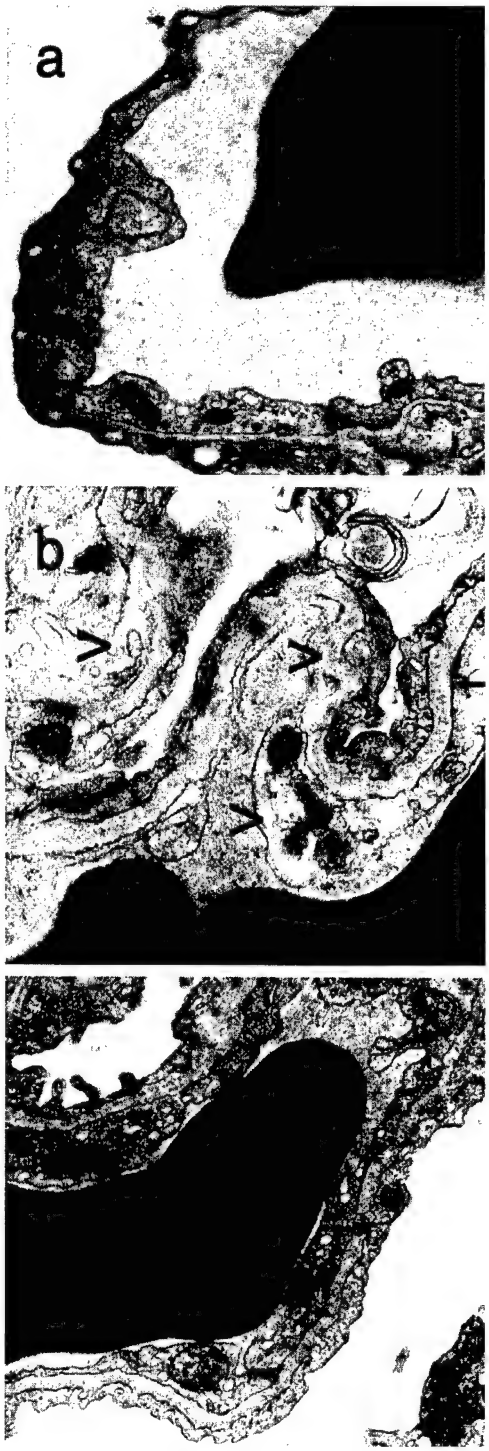

Figure 4.
Ultrastructure of the lungs of mice given IL-2 or IL-2 + L-NAME therapy. a, control; b, IL-2; c, IL-2 + L-NAME; magnification, ×17,120. Basement membrane is thick (←) and discontinuous in IL-2–treated mice. Endothelial as well as pneumocyte type I cells are severely damaged. There is also swelling of endothelial cells as well as pneumocyte type I. < indicates an area of blood-air barrier showing such damage. Basement membrane is continuous and thin at the thin part of the capillary (*) in IL-2 + L-NAME-treated animals. Endothelial cells, although in some cases remaining swollen, are never detached from their basement membrane in these mice.

induced iNOS expression in endothelial cells of capillaries surrounding the fibers of intercostal muscles of the anterior thoracic wall (Fig. 8b). The addition of L-NAME therapy, although eradicating all NOS activity from the anterior thoracic wall (Fig. 6), did not influence the expression or distribution of iNOS enzyme. After two rounds of IL-2 with or without L-NAME, there

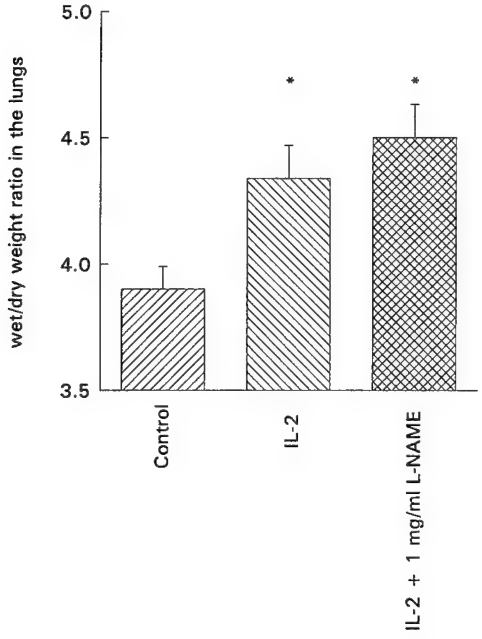

Figure 5.
Water content of the lungs after the second round of IL-2 ± L-NAME therapy. Data represent mean + SE ($n = 5$). * indicates significant difference from control ($p < 0.05$). Addition of L-NAME therapy did not influence IL-2–induced pulmonary edema after the second round of therapies.

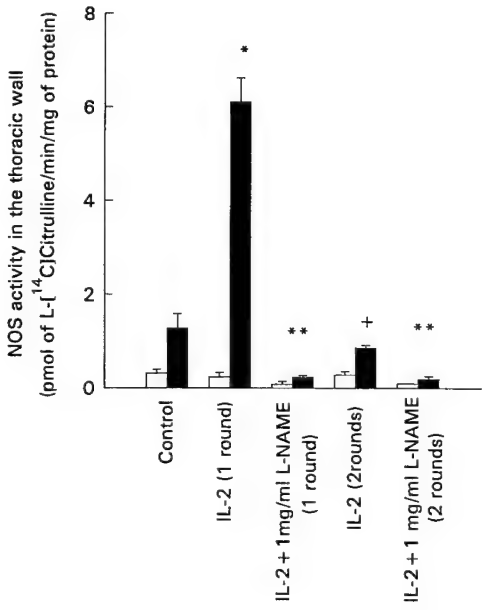

Figure 6.
NOS activity in the thoracic wall after one or two rounds of IL-2 ± L-NAME therapy. *, one round of IL-2 therapy significantly ($p < 0.05$) increased Ca^{2+}-independent NOS activity in the anterior thoracic wall. **, addition of L-NAME therapy abolished both forms of NOS activity, after either one or two rounds of therapy. +, there was no significant iNOS activity induced by the second round of IL-2 therapy. □, Ca^{2+}-dependent activity; ■, Ca^{2+}-independent activity.

was stronger staining for iNOS enzyme present in the endothelial cells of capillaries surrounding the fibers of intercostal muscles, as well as a punctate staining of some muscle fibers (Fig. 8, d and e). Electron micro-

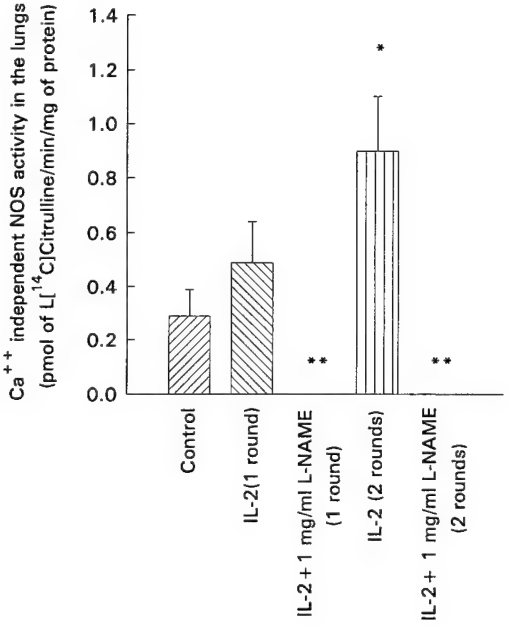

Figure 7.
Ca^{2+}-independent iNOS activity in the lungs after one or two rounds of IL-2 ± L-NAME therapy. *, IL-2 therapy induced significant ($p < 0.05$) iNOS activity in the lungs after the second round of therapy. **, L-NAME therapy abolished all iNOS activity in the lungs after either one or two rounds of therapy.

scopic immunocytochemistry revealed that iNOS enzyme was present in the sarcoplasm of some intercostal muscles (Fig. 9a) and in the cytoplasm of endothelial cells of capillaries and small arteries (Fig. 9b).

Lungs. Although NOS activity in the lungs was significantly induced by two rounds of IL-2 therapy (Fig. 7), there was no significant difference in iNOS staining between the lungs of control and treated mice (data not presented).

Spleen. iNOS-positive macrophages were scarce in the spleens of normal mice but abundant in the spleens of IL-2 or IL-2 + L-NAME-treated animals after one or two rounds of therapy (Fig. 10). After one round of therapy, iNOS positive macrophages were present mostly in the red pulp of the spleens of IL-2–treated mice, whereas in mice subjected to IL-2 + L-NAME, they had accumulated at the periphery of the white pulp. After two rounds of therapy, iNOS-positive macrophages accumulated within the white pulp of IL-2–treated animals and were most numerous at the periphery of the white pulp of IL-2 + L-NAME-treated animals.

Discussion

The present study revealed that IL-2 therapy in healthy mice induced significant Ca^{2+}-independent (ie, inducible) NOS activity in the lungs and the thoracic wall concomitant with the induction of pulmonary edema

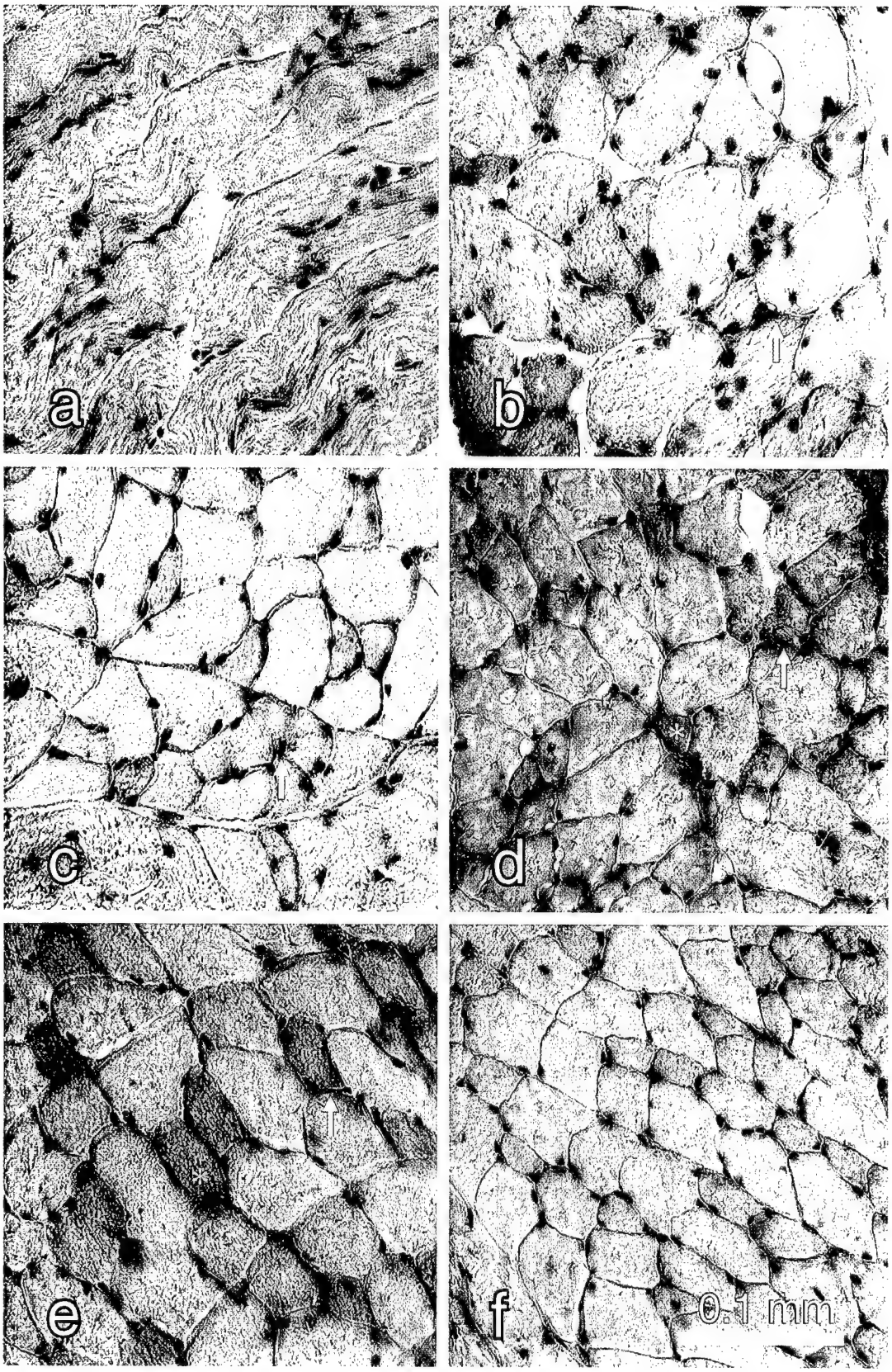

Figure 8.

Immunostaining with a mouse monoclonal antibody against iNOS (a to e), and a negative control antibody of the same isotype (f) in the anterior thoracic wall after one or two rounds of IL-2 ± L-NAME therapy, lightly counterstained with hematoxylin. a, control mice; b, IL-2, 1 round; c, IL-2 + L-NAME, 1 round; d, IL-2, 2 rounds; e and f, IL-2 + L-NAME, 2 rounds. Strong endothelial cell staining (↑) is present in all IL-2–treated groups. Staining of endothelial cells appears stronger after two rounds of therapy, when staining is also noted in some muscle fibers (*). There was no positive immunostaining in the presence of a negative control antibody of the same isotype in any tissue of the anterior thoracic wall after one or two rounds of IL-2 ± L-NAME therapy (f).

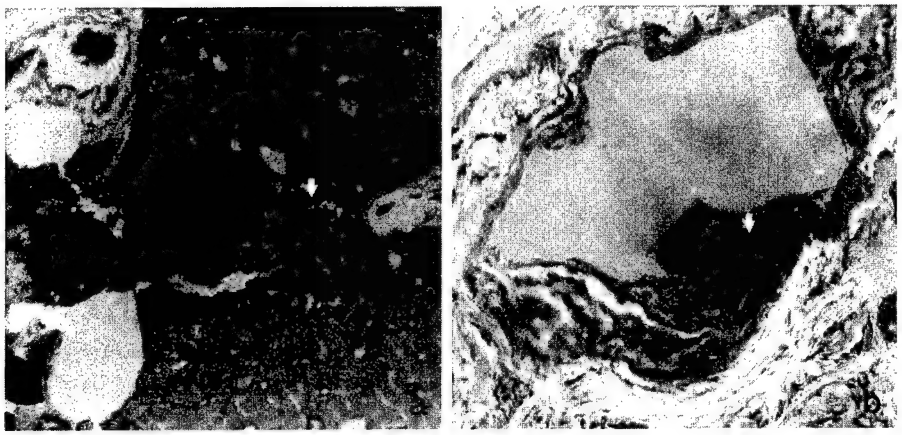

Figure 9.
Electron microscopic immunolocalization of iNOS protein in the intercostal muscle fibers (a) and arteriole (b) in the anterior thoracic wall from a mouse treated with two rounds of IL-2. iNOS immunoreactivity was located in the sarcoplasm of some intercostal muscles (a) as well as in the cytoplasm of endothelial cells (b) of arterioles. Magnification, ×5715.

and pleural effusion. The addition of oral L-NAME therapy ameliorated these more severe symptoms of IL-2–induced capillary leakage and coincided with the abrogation of NOS activity in these tissues, suggesting that a high local NOS activity was instrumental in the pathogenesis of capillary leakage.

Pleural effusion after one round of IL-2 therapy was accompanied by an induction of highly active NOS enzyme in the anterior thoracic wall. The enzyme was mostly localized in the endothelium of the capillaries surrounding the intercostal muscle fibers. Because the parietal pleura is supplied by the vessels that also supply the thoracic wall, it is likely that this local source of NO contributed to the high NO level in the pleural fluid. There was no pleural effusion in mice after two rounds of IL-2 therapy, confirming our previously reported findings (Orucevic and Lala, 1996c, 1996d). Interestingly, there was also a lack of significant induction of iNOS activity in the thoracic wall at this time, which reconfirms the association of pleural effusion with local NOS activity. The findings of low NOS activity can, perhaps, best be explained by the feedback inhibition of NOS activity by high NO levels, as reported by Moncada's group for a macrophage cell line in vitro (Assreuy et al, 1993). Subsequently, it was demonstrated by Luss et al (1994) that NO not only exerted feedback inhibition of NOS activity but also decreased the level of iNOS protein expression, most likely by inhibiting translation of the iNOS protein. Surprisingly, however, an abundance of immunoreactive iNOS protein (both after the second round of IL-2 or IL-2 + L-NAME) was still detected in the anterior thoracic wall in the present study. Electron microscopic immunocytochemistry confirmed that the protein was present within the endothelium of capillaries surrounding the intercostal muscle fibers and also some muscle fibers. Currently, it is not possible to give an explanation for the detection of iNOS protein in the absence of iNOS activity after the second round of IL-2. This paradox remains to be resolved by further quantitative studies, eg Western blot analysis of the iNOS protein.

IL-2–induced pulmonary edema was present after both rounds of IL-2 therapy and was accompanied by a significant NOS activity in the lungs. Addition of L-NAME therapy during either round abolished NOS activity but reduced pulmonary edema only after first round of IL-2 therapy. Thus, one would assume that NO induction as well as other mechanisms may be responsible for the presence of pulmonary edema after the second round of IL-2 therapy, so that inhibition of NO synthesis with L-NAME was not enough to prevent IL-2–induced pulmonary edema. It is likely that a direct LAK-cell-mediated injury to the pulmonary endothelium was greater after the second round of IL-2.

Although L-NAME was effective in abolishing the NOS activity in the lungs and the thoracic wall after the second round of IL-2 therapy, the reduction in serum NO level was not significant. This may indicate that L-NAME did not totally eradicate iNOS activity in all cells that contributed to the serum NO level after the second round of therapy. It remains to be investigated whether more selective iNOS inhibitors would provide a better therapeutic efficacy against IL-2–induced capillary leakage.

A significant increase in the thickness of the interalveolar connective tissue space (as measured by computerized histomorphometry) accompanied IL-2–induced pulmonary edema. Histologic analysis revealed marked lymphocyte infiltration similar to those showed by Parhar and Lala (1987) and Dubinett et al

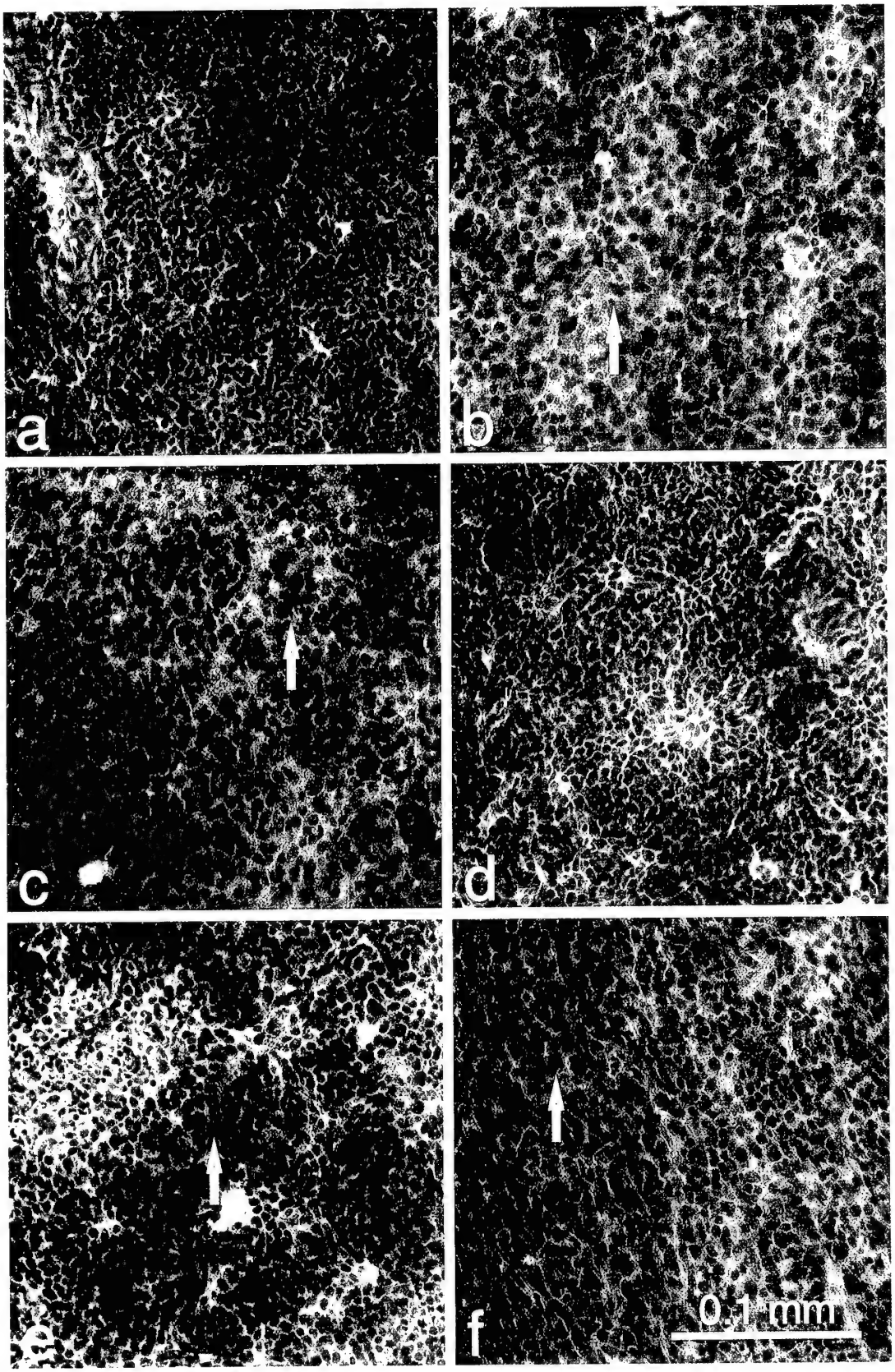

Figure 10.

Immunostaining with a mouse monoclonal antibody against iNOS (a to c, e and f) and a negative control antibody of the same isotype (d) in the spleen after one or two rounds of IL-2 ± L-NAME therapy, counterstained with hematoxylin. a, control; b, IL-2, 1 round; c and d, IL-2 + L-NAME, 1 round; e, IL-2, 2 rounds; f, IL-2 + L-NAME, 2 rounds. (↑) Staining is present in a substantial population of cells (identified as macrophages at higher magnifications) in the spleens of all IL-2–treated groups. There was no positive immunostaining in the presence of a negative control antibody of the same isotype in the spleen after one or two rounds of IL-2 ± L-NAME therapy (d).

(1994). Ultrastructural analysis revealed that IL-2–induced pulmonary edema was associated with a major distortion in the capillary architecture of the lungs. The changes included swelling or damage of endothelial cells and type I pneumocytes, thickening of the basement membrane of the thin portion of the capillaries, or herniation of the thin segment of endothelial cells into the vessel lumen because of the accumulation of fluid between the plasma membrane and the basement membrane. Similar findings were described in the lungs of rats and rabbits treated with IL-2 (Renzi et al, 1991; Goldblum et al, 1990). Dubinett et al (1994) suggested that IL-2–induced tumor necrosis factor (TNF) α played a central role in mediating the pulmonary vascular leakage, because IL-2 upregulated the in situ expression of both the TNFα mRNA and the protein in the lungs, whereas administration of a soluble TNFα receptor significantly reduced IL-2–induced pulmonary edema. The present study revealed that NO inhibition with L-NAME reduced IL-2–induced pulmonary edema, concomitant with a significant reduction of the thickness of interalveolar spaces. This was associated with ultrastructural evidence of significant restoration of capillary architecture in the lungs. Because TNFα caused endothelial cell damage in vitro through the production of NO (Palmer et al, 1992; Estrada et al, 1992), it is reasonable to postulate that IL-2–induced TNFα production in vivo may have been instrumental in high NO production, leading to pulmonary edema.

Macrophages immunoreactive for the iNOS protein became abundant in the spleen of IL-2- or IL-2 + L-NAME-treated animals after either one or two rounds of therapies. It appeared that there were more macrophages stained in the spleens of mice treated with IL-2 + L-NAME, than with IL-2 alone. Luss et al (1994) reported that chronic inhibition of NO production can result in an increase in iNOS mRNA and protein levels in iNOS expressing cells, although such iNOS may remain inactive because of the presence of L-NAME. We have earlier shown (Orucevic and Lala, 1996b) that in vivo LAK-cell activation in splenocytes of IL-2 + L-NAME-treated mice was significantly higher than that in IL-2–treated animals, suggesting that the IL-2–induced increase in NOS activity followed by increased NO production within the spleen interfered with optimal LAK cell activation. Whether a cessation of L-NAME therapy would lead to a rebound in NO production in the presence of high iNOS protein levels and whether this would influence splenocyte cytotoxicity remains to be investigated.

In conclusion, the present study revealed that a rise in NO metabolites in the tissue fluids was associated with a high local NOS activity as well as expression of iNOS protein during IL-2 therapy. A reduction of NOS activity, but not necessarily the expression of iNOS protein, was a good indicator of therapeutic effectiveness in the amelioration of capillary leakage with L-NAME therapy. It is likely that NOS inhibitors can be useful adjuncts to IL-2 therapy of cancer and infectious diseases.

Materials and Methods

Mice

C3H/HeJ female mice (7 to 8 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, Maine). Animals were fed standard mouse chow, provided with water ad libitum, and kept on a 12-hour light/dark cycle. Animal care was in accord with the guidelines set out by the Canadian Council on Animal Care.

Interleukin-2

Recombinant, highly purified human IL-2 (lot LQP-046) was kindly provided by the Chiron Corporation (Emeryville, California). The specific activity was 3×10^6 Cetus Units (CU) or 18×10^6 IU/mg of IL-2. The lyophilized IL-2 (1.2 mg/vial) was first reconstituted with 1 ml of distilled water. RPMI–1640 medium (ICN Biomedicals, Inc., Costa Mesa, California) was used to dilute it further to obtain 15,000 CU in 0.1 ml per injection. Previously, we found this dose to induce capillary leakage (Orucevic and Lala, 1996a, 1996d). The reconstituted material was stored at 4°C up to 1 day.

L-NAME

L-NAME obtained from Sigma Chemical Company (St. Louis, Missouri) was added to the animals' drinking water to provide concentrations of 1 mg/ml. We previously found this dose to significantly reduce IL-2–induced capillary leakage and to increase IL-2–induced NO production in healthy mice (Orucevic and Lala, 1996d).

Experimental Design

Healthy mice (n = 20 per group) received one of the following treatments: nothing; 15,000 CU of IL-2 alone given ip every 8 hours for 10 injections; or IL-2 + L-NAME (1 mg/ml of drinking water starting 1 day before IL-2 therapy). Mice (n = 8 per group) were killed 1 hour after the last IL-2 injection to measure NOS activity in the lungs and anterior thoracic wall, as well as tissue distribution of iNOS enzyme in the lung,

intercostal muscles, and spleen. Water content in the pleural cavities (known to be a reliable marker of IL-2–induced CLS in mice; Orucevic and Lala, 1996a, 1996d) and NO_2^- +NO_3^- levels in the serum and pleural effusion were also measured. Structural changes of the lungs during IL-2 therapy were examined using light and electron microscopy. All of the above parameters were examined to establish the relationship between NOS activity and protein expression within the tissues and the degree of IL-2–induced CLS.

The remainder of the mice (n = 12 per group) were given a second round of IL-2, which started 6 days after the first round and followed the same schedule, doses, and route of administration as the first round. L-NAME was also given in drinking water (1 mg/ml, starting 1 day before the second round of IL-2). Mice were killed at the end of the second round of IL-2 (1 hour after the last IL-2 injection) to measure the same parameters as the first round. The differences in NOS activity or iNOS protein expression between one and two rounds of IL-2 therapy were then examined.

Measurement of Pleural Effusion, Pulmonary Edema, and NO Production

For measurement of water content, the left lung was recovered from animals, and its wet weight was recorded. The lungs were frozen at −80°C and then freeze dried to constant weight in a freeze-drying system (Labconco Corporation, Kansas City, Missouri). Dry weights were measured, and wet to dry weight ratio of the lungs was calculated (Orucevic and Lala, 1996c, 1996d).

The volume of liquid from both pleural cavities was measured directly by complete aspiration with a 1-ml syringe (Orucevic and Lala, 1996c, 1996d).

Samples of serum and pleural effusion were collected after the end of treatments to measure NO_2^- and NO_3^-, the principal metabolites of NO (Moncada and Higgs, 1993; Kelm et al, 1992). Cadmium filings were used for the conversion of NO_3^- to NO_2^- (Davison and Woof, 1978); Griess reaction was used to measure NO_2^- (Green et al, 1982). The basal concentrations of NO_2^- were not detectable; we were, however, able to obtain measurable amounts of NO_2^- from our samples by reducing the NO_3^- to NO_2^-. The absorbance was read at 543 nm in a DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, California). The final concentration of nitrite in pleural effusion and serum was calculated from a sodium nitrite standard curve, which was linear from 0 to 90 μM nitrite (Orucevic and Lala, 1996c, 1996d).

Light and Electron Microscopy of the Lungs

Lungs were fixed in 2.5% glutaraldehyde in 0.1 M/l of sodium cacodylate buffer by immersion, postfixed in 1% osmium tetroxide in 0.1 M/l cacodylate buffer, infiltrated, and embedded in plastic (epoxy resin). Semithin sections (0.5 μm) were stained with toluidine blue for light microscopic analysis. Thin sections (90 nm) were stained with lead citrate and uranyl acetate (Hunter, 1993) and examined with an electron microscope. Light microscopic image analysis (Jandel Scientific) was used to establish the degree of pulmonary edema (induced by IL-2 therapy and abrogated by L-NAME therapy) and to calculate the percentage of area occupied by the connective tissue of the lungs versus the percentage of area occupied by the air spaces of the lungs. Ultrastructural changes in the lung morphology (interalveolar septa as well as blood-air barrier) were analyzed through the use of electron microscopy. Alterations in endothelial and epithelial cell morphology, endothelial continuity, thickness of the endothelial and epithelial basement membranes, and the characteristics of migratory cells in the interalveolar septa were analyzed.

Measurement of NOS Activity

The lung and anterior thoracic wall (all tissues except the skin) were collected from mice receiving nothing or one or two rounds of IL-2±L-NAME, snap-frozen in liquid nitrogen, and stored at −70°C until assayed for NOS activity. Assay of NOS was performed as described by Thomsen et al (1995). All reagents were obtained from Sigma unless otherwise stated.

Frozen tissue (n = 5 per treatment group) was homogenized (with a polytron) in 5 volumes of a buffer containing 20 mM HEPES, 0.1 mM EDTA, 0.2 mM sucrose, 5 mM DL-dithiothreitol (Boehringer Mannheim, Laval, Quebec), 10 μg/ml each of leupeptin (Boehringer Mannheim) and soybean trypsin inhibitor, and 1 μg/ml pepstatin. The homogenates were then centrifuged at 10,000g at 4°C for 30 minutes. Endogenous arginine from obtained supernatants (cytosol plus microsomes) was removed by addition of 1:5 ratio of cation:exchange resin (Dowex 50×8 to 400), followed by short centrifugation (1 minute, 10,000g). NOS in the supernatants was measured by conversion of L-[U-^{14}C] arginine (Amersham Life Science, Clearbrook, Illinois) to [U-^{14}C] citrulline at 37°C for 10 minutes, as described by Salter et al (1991). In brief, 100 μl of substrate (uninhibited substrate) containing 10 μM tetrahydrobiopterin, 2.5 mM of DL-dithiothreitol, 4000 U/ml calmodulin, 250 μM $CaCl_2$, 0.5 mg/ml BSA, 125 μM NADPH, 1500 pmol arginine (cold and hot),

and 100 μM of L-Citrulline in HEPES buffer was incubated with 50 μl of enzyme (supernatant) for 10 minutes in a water bath set at 37°C. The reaction was stopped with the addition of 500 μl of Dowex and 1000 μl of distilled water. Twenty to thirty minutes later, when Dowex had settled at the bottom of the tube, and theoretically all of the ^{14}C-L-arginine had adhered to Dowex, 975 μl of clear upper phase containing only ^{14}C-L-citrulline was mixed with 3 ml of scintillation cocktail (Amersham Canada Ltd., Oakville, Ontario, Canada) and counted on the scintillation counter.

NOS activity in the citrulline assay was calculated from the difference of counts per minute between a substrate and appropriate blank. In these incubations, blank was achieved by addition of competitive NOS inhibitor, whereas Ca^{2+} chelator, ethyleneglycol-bis (36 -aminoethyl ether)-*N, N, N', N*-tetraacteic acid (EGTA), served to block Ca^{2+}-dependent NOS activity. Thus, the activity of Ca^{2+}-dependent enzyme was calculated from the difference between the [U-^{14}C]citrulline generated from uninhibited samples and samples containing 1 mM EGTA. The activity of the Ca^{2+}-independent enzyme was calculated from the difference between samples containing 1 mM EGTA and samples containing both 1 mM EGTA and 1 mM NMMA. The total protein content of tissue supernatant was determined spectrophotometrically (BioRad assay, Bio-Rad, Richmond, California), and final NOS activity was expressed as pmol of citrulline/minutes/mg of protein.

Immunocytochemical Localization of iNOS Protein in Tissues Analyzed by Light and Electron Microscopy

Unfixed samples of lungs, intercostal muscles, and spleen were frozen in cryopreservative optimal cutting temperature in OCT (optimal cutting temperature) liquid nitrogen-cooled isopentane, sectioned on a cryostat (20 μm), and melted directly onto glass slides. Sections were fixed in 10% buffered formalin, endogenous peroxidase activity was blocked with 3% H_2O_2 in absolute methanol, and tissue was permeabilized with 0.25% Triton X-100 in PBS. After washing (3 × 5 minutes PBS), 10% normal horse serum in 0.2% BSA was added onto the sections as blocking serum. This was followed by the addition of a complex of a primary (anti-mac NOS, mouse monoclonal antibody against mouse macrophage iNOS; Transduction Laboratories, Lexington, Kentucky) and a secondary horse anti-mouse biotinylated antibody (Dimension Laboratories, Mississauga, Ontario, Canada) (1:50 and 1:200 dilution, respectively). The complex was made by shaking primary and secondary antibody overnight at 4°C, followed by the addition of heat-inactivated normal mouse serum to a final concentration of 0.2% (v/v) 2 hours before adding the complex to the sections. This procedure, described by Hierck et al (1994), was proven to significantly reduce the high background usually resulting with mouse monoclonal antibodies when applied to mouse tissues. High background is the result of nonspecific binding of secondary anti-mouse Ab to the immunoglobulins normally present in mouse tissues. In the above-mentioned procedure, a complex of primary and secondary antibody is made in a test tube; normal mouse serum is then added to bind to all unbound secondary Ab so that, theoretically, when added, the only possible binding of the complex is to the iNOS antigen present in the tissues. As a negative control, anti-iNOS Ab was replaced with a mouse monoclonal antibody against *Aspergillus niger* glucose oxidase (DAKO; supplied by Dimension Laboratories), an enzyme that is neither present nor inducible in mammalian tissues. Sections were incubated with the complex for 4 hours and washed; avidin-biotin peroxidase complex substrate was then added, followed by diaminobenzidine chromogen treatment. Sections were counterstained with hematoxylin, mounted with aqua-mount, and analyzed for the presence of iNOS protein.

For electron microscopic immunocytochemistry, a slightly modified method described by Xue et al (1996) was used. Cryostat sections immunostained for iNOS on glass slides, as described above, were further processed for viewing in the electron microscope. After incubation in diaminobenzedine substrate, sections were rinsed in distilled water and postfixed with 1% osmium tetroxide. Slides were then placed in a slide transport container, dehydrated in ethanol, and infiltrated with epoxy resin (Polybed 812, Polysciences, Warrington, Pennsylvania). Slides were then removed from the transport containers and placed section-side-up on a horizontal surface. Embedding capsules were placed over the sections and filled with fresh resin; the resin was polymerized overnight in an oven at 60°C. Sections polymerized inside embedding capsules were removed from the surface of the glass slides by repeated plunge-thawing in liquid nitrogen. Ultrathin sections were then prepared and counterstained for 10 seconds in 3% uranyl acetate in 30% alcohol.

Image Analysis

Image analysis was performed using an IBM-compatible computer and two computer packages: Jandel Scientific Mocha Image Software and Northern Exposure, version 2.3. Photomicrographs were pre-

pared in a digital darkroom using Adobe Photoshop (Mountainview, California) and Corel Draw software programs (Corel Corporation, Ottawa, Ontario, Canada). Prints were generated with a Tektronic Phaser 440 printer (Wilsonville, Oregon).

Statistical Analysis

Data were subjected to ANOVA using Microstat Statistics Package (Ecosoft, Inc., Indianapolis, Indiana). A one-way ANOVA test was used for normal distribution, and a Kruskal-Wallis test was used for skewed distributions. Newman-Keuls multiple range test or nonparametric multiple range test was used to further determine which means or sums of ranks were different from one another (Zar, 1974). $p < 0.05$ was considered significant.

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A2

Role of nitric oxide in tumor progression: Lessons from experimental tumors

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Key words: nitric oxide, invasion, metastasis, angiogenesis, mammary tumor

Abstract

Nitric oxide (NO), a potent biological mediator, plays a key role in physiological as well as pathological processes, including inflammation and cancer. The role of NO in tumor biology remains incompletely understood. While a few reports indicate that the presence of NO in tumor cells or their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, a large body of clinical and experimental data suggest a promoting role of NO in tumor progression and metastasis. We suggest that tumor cells capable of very high levels of NO production die *in vivo,* and those producing or exposed to lower levels of NO, or capable of resisting NO-mediated injury undergo a clonal selection because of their survival advantage; they also utilize certain NO-mediated mechanisms for promotion of growth, invasion and metastasis. The possible mechanism(s) are: (a) a stimulatory effect on tumor cell invasiveness, (b) a promotion of tumor angiogenesis and blood flow in the tumor neovasculature, and (c) a suppression of host anti-tumor defense. In this review, we discuss these mechanisms on the basis of data derived from experimental models, in particular, a mouse mammary tumor model in which the expression of eNOS by tumor cells is positively correlated with invasive and metastatic abilities. Tumor-derived NO was shown to promote tumor cell invasiveness and angiogenesis. The invasion-stimulating effects of NO were due to an upregulation of matrix metalloproteases and a downregulation of their natural inhibitors. Treatment of tumor-bearing mice with NO-blocking agents reduced the growth and vascularity of primary tumors and their spontaneous metastases. We propose that selected NO-blocking drugs may be useful in treating certain human cancers either as single agents or as a part of combination therapies.

I. Introduction

Progression of solid tumors is a multistage process involving genetic changes in tumor cells that provide selective advantages for growth, invasion, and metastasis due to tumor-derived (autocrine) or host-derived (paracrine) signals capable of promoting these events. Growth of primary as well as metastatic tumors can be facilitated by direct proliferation-stimulating events such as a perpetuation of positive growth-regulating signals, e.g., activation of certain protooncogenes which serve as receptors for proliferation-stimulating growth factors, or production of proliferation-stimulating growth factors. This can also result from a loss of negative growth-regulating signals, e.g., inactivation of certain tumor suppressor genes involved in cell cycle control or receptors for proliferation-blocking growth factors [1, 2]. Tumor growth can also be facilitated indirectly by promotion of tumor angiogenesis and tumor blood flow [3].

For tumor cells to invade into surrounding normal tissues or metastasize to a distant site, a number of steps must be completed successfully [4]. First, tumor cells must bind to one or more constituents of the basement membrane or extracellular matrix

(ECM) via cell surface integrins or non-integrin receptors. This binding is more than an adhesive event; it can also lead to transduction of signals that may facilitate invasion [5]. Second, tumor cells must degrade basement membrane and ECM constituents; this step is facilitated by the production of active matrix degrading enzymes in excess of natural inhibitors of these enzymes [4, 6]. Third, tumor cells must migrate through the degraded ECM. This step is facilitated by the anchoring of cells to the ECM by appropriate integrin(s) [7], and by migration-promoting cell-ligand interactions [8]. Finally, for metastasis to occur, tumor cells must intravasate and survive within the blood vessels or lymphatics, and then extravasate and seed at distant locations. Recent work utilizing live videomicroscopy has demonstrated that even after successful extravasation and seeding, many tumor cells may either die or remain quiescent for a significant period [9]. Thus, successful metastasis often requires additional autocrine/paracrine growth or angiogenesis-stimulating signals at the new site. Similar steps when successfully repeated, may allow metastatic tumors to remetastasize to newer sites.

In the present article we shall discusss the contributory role(s) of nitric oxide (NO) in tumor progression and metastasis in the context of the above events which can influence growth, invasion or metastasis in experimental tumor models.

During the last decade, following the discovery [10] that NO accounts for the full biological activity of endothelium-derived relaxing factor (EDRF) [11], NO has been shown to be produced by many mammalian cells and responsible for numerous physiological functions. These include vasodilation, inhibition of platelet aggregation, modulation of neurotransmission, and mediation of injury by macrophages to bacteria, parasites and tumor cells [12–17]. On the other hand, sustained high levels of NO production in the body can also lead to pathological injuries mediated by NO or its metabolites [18]. NO production depends on conversion of the amino acid L-arginine to L-citrulline by a family of enzymes named NO synthases (NOS) [19–20]. Three isoforms have been identified so far. The endothelial type NOS (eNOS) is a constitutive, Ca^{++} and calmodulin-dependent form of the enzyme, expressed by many cells including endothelial cells, myocardial cells and pyramidal cells of the hippocampus. The neuronal type NOS (nNOS) is also constitutive, Ca^{++} and calmodulin-dependent, and is expressed by certain cells including neurons of the central nervous system, the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells. Inducible type NOS (iNOS) is Ca^{++} and calmodulin-independent, and is expressed by macrophages in many mammalian species, endothelial cells, hepatocytes, cardiac myocytes, chondrocytes and many other cells following stimulation by inflammatory cytokines and/or bacterial endotoxin [20–23]. The expression of iNOS is high in activated rodent macrophages and endothelial cells, and poor in human macrophages [24].

NO is often an important component of the chemical microenvironment of tumors, produced either by tumor cells, endothelial cells in the tumor microvasculature or macrophages and stromal cells within the tumors. Because of its lipophilic nature, NO can rapidly cross cell membranes and enter intracellular compartments to exert its action, even when produced by a neighbouring cell. Thus, it can mediate interactions between tumor cells and host cells. The functional role of NO in tumor biology is complex and remains to be fully defined. While a small number of reports indicate that the presence of NO in tumor cells or in their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, numerous clinical and experimental studies suggest a promoting role of NO in tumor progression and metastasis. In this review, we discuss these two apparently conflicting views and suggest that the opposing effects of NO may depend on two important variables: the levels of NO production and the genetic makeup of tumor cells. We suggest that in a heterogeneous population of tumor cells, clonal evolution favors those capable of resisting NO-mediated injury. In addition, many tumor cells may also utilize NO in their microenvironment, facilitating some of the steps required for tumor growth, invasion and metastasis; this will be illustrated from our own studies using spontaneous C3H/HeJ mouse mammary adenocarcinomas and their clonal derivatives as an experimental model for tumor progression and metastasis.

II. Association between NO and tumor growth

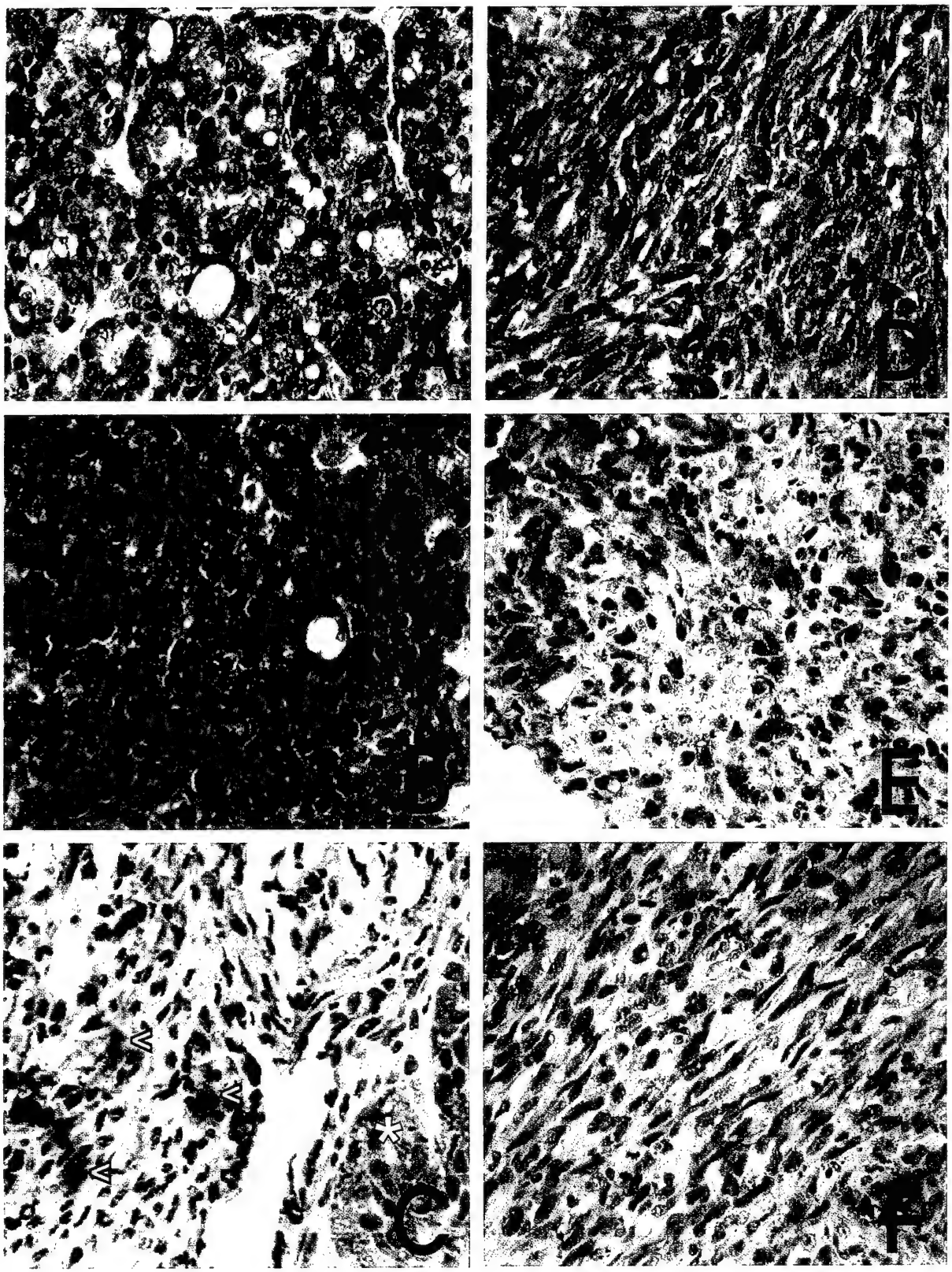
The genotoxic role of NO in promoting carcinogenesis is well recognized. The underlying mechanisms are the subject matter of another article (by Felley-Bosco) in this issue. Chronic exposure of cells to NO can result in multiple genetic changes which may underlie histological changes such as metaplasia and the progression of metaplasia into neoplasia. The presence of eNOS in breast apocrine metaplastic cells of fibrocystic disease in the human [25] and iNOS in macrophages within the hyperplastic stromal tissue of a rat model of Barret's esophagus [26] are believed to promote the progression of metaplastic epithelia into carcinomas.

Human tumor materials, in general, have provided the strongest link between NO production and disease progression. While the functional implications of elevated serum NO levels observed in many cancer patients [27] remain unexplored, a number of reports indicate a contributory role of NO to tumor progression. An abundant expression of NOS, as well as NOS activity, has been positively correlated with the degree of malignancy in human ovarian and uterine cancers [28], central nervous system tumors [29], and breast cancer [30]. Contributing to the elevated NOS activity are constitutive form(s) in tumor cells [28, 29] and/or tumor endothelial cells [29], and the inducible form in the tumor endothelial cells [29] and/or tumor associated macrophages [30].

The relationship of NO to human colonic tumor progression remains controversial. Histochemical localization of NAD(P)H diaphorase enzyme, NOS activity and NOS expression in the human colonic mucosa, polyps and carcinomas suggest an inverse relationship between the enzymes and colonic tumor progression [31, 32]. In contrast, studies of NOS gene expression and NOS activity in a panel of human colonic adenocarcinoma cell lines revealed that all expressed mRNA for the eNOS gene, and some exhibited significant NOS activity [33]. Evidently, more studies are needed in human colonic tumors.

NOS expression has been examined in a number of experimental tumor models. An abundant expression of iNOS by cells of the tumor vasculature has been implicated in the promotion of tumor growth both in murine [34] and rat [35] tumors. eNOS expression by tumor cells is positively correlated with invasiveness and metastasis in a murine mammary adenocarcinoma model, to be described later.

Numerous studies in animal models have provided direct evidence for a stimulatory role of NO in tumor progression. In a rat colonic adenocarcinoma model showing iNOS expression in the tumor vasculature, treatment with N^G-Nitro-L-arginine methyl ester (L-NAME), a potent NOS inhibitor, reduced NO production and tumor growth [35]. Similarly, anti-tumor and anti-metastatic effects of two NOS inhibitors N^G-methyl-L-arginine (NMMA) and L-NAME were observed in our laboratory using a mouse mammary adenocarcinoma model [36, 37], in which tumor cells expressed eNOS. Recently, Edwards *et al.* [38] observed that NO production induced by lipopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine breast cancer cells inhibited cell growth *in vitro*, but stimulated tumorigenesis and experimental lung metastasis *in vivo*. Finally, engineered expression of murine iNOS in a human colonic adenocarcinoma cell line resulting in continuous, moderate levels of NO production *in vitro*, was associated with increased tumor growth and vascularity *in vivo* following transplantation in nude mice [39]. These findings of a facilitatory role of NO in tumor growth and metastasis are in contrast with those reported for murine K1735 melanoma cell lines, in which the level of iNOS expression was inversely correlated with their ability for experimental metastasis [40]. Furthermore, engineered overexpression of iNOS in an iNOS-deficient melanoma line suppressed tumorigenic and metastatic abilities *in vivo* because of NO-mediated cytostasis and apoptosis [41]. Two explanations may be offered for these apparently conflicting results. First, very high NO levels (such as those produced by the iNOS overexpressing murine melanoma line) can be detrimental to tumor cell survival. Indeed, the iNOS overexpressing melanoma line had poor survival in the absence of NOS inhibitors *in vitro* and *in vivo* [41]. Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis, certain tumor cells can not only re-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sist NO-mediated injury, but also utilize NO to facilitate tumor progression and metastasis. These possibilities will be discussed later in more detail.

III. C3H/HeJ spontaneous mammary adenocarcinoma and its clonal derivatives: a model for tumor progression

Approximately 90% of C3H/HeJ female retired breeder mice develop mammary tumors during their lifespan [42]. Despite extensive variation in the site (anywhere in the mammary line) or the time (6 months to 2 years) of tumor appearance, most tumors are highly vascular and exhibit histological features of invasive adenocarcinomas with a pseudoglandular architecture. In general, animals demonstrating spontaneous primary tumors develop eventual metastasis in their lungs. Lung metastasis may occasionally be present even in the absence of a visible or palpable primary tumor [86]. Tumor development in these mice requires the proviral form of the mouse mammary tumor virus (MMTV), which is transmitted via the mother's milk and integrated in the developing mammary tissue of the female offspring. This leads to tumorigenesis owing to insertional mutagenesis of certain important growth-regulating genetic loci which serve as the proviral integration sites [43, 44].

Clonal derivatives of spontaneous mammary tumors exhibited extensive heterogeneity in growth rates observed *in vitro* and *in vivo*, and metastasis formation *in vivo* following subcutaneous transplantation in syngenic mice [45]. Two clones derived from a single spontaneous tumor differed markedly in their abilities for spontaneous lung metastasis from a primary subcutaneous transplant site; C10 was poorly metastatic, and C3 was highly metastatic. However, because the metastatic ability of C3 declined after several years of *in vitro* passage [46], C3 cells were subjected to an *in vivo* selection pressure of 5 cycles of subcutaneous to pulmonary passage, yielding highly metastatic cells [47]. The resulting cell line, designated C3L5, has since maintained a very high ability for spontaneous lung metastasis from subcutaneous sites. The data presented below relate to C3H/HeJ spontaneous mammary tumors as well as the high and low metastatic clonal derivatives, C3L5 and C10, respectively.

IV. Relationship of NO production to tumor growth and metastasis in the C3H/HeJ mammary tumor model

IV.A. NOS expression in spontaneous (primary and metastatic) mammary tumors (Lala, Orucevic and Hum, unpublished)

Random examination of spontaneously developing mammary tumors harvested at 12 weeks of tumor age revealed immunohistochemical evidence of eNOS expression in tumor cells (Figure 1A and B) and iNOS expression in certain macrophages in the tumor stroma (Figure 1C). Endothelial cells in the tumor vasculature were eNOS positive.

In the primary tumors, a heterogenous pattern of eNOS staining was noted; tumor cells in pseudoacinar formation were either strongly positive or negative (Figure 1A). However, the lung metastatic nodules were composed primarily of strongly eNOS positive cells (Figure 1B), suggesting a positive cor-

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Figure 1. Immunostaining patterns for eNOS (A, B, D and E) and iNOS (C) in 12 week old spontaneous (A, B and C) and 3·5 week old transplanted C3L5 (D, E) mammary tumors at the primary (A, C, and D) and metastatic (B, E) sites. Positive immunoreactivity is indicated by brown staining. Blue staining (nuclei, and to a minor extent cytoplasm) is due to counterstaining with hematoxylin.

A Spontaneous primary tumor showing eNOS positive as well as eNOS negative tumor cells arranged in pseudoacinar clusters.

B Lung metastasis of the tumor A showing strong eNOS positivity in most tumor cells.

C Spontaneous primary tumor showing iNOS positive macrophages (<) in the tumor stroma. Tumor cells (*) were iNOS negative.

D Subcutaneous C3L5 primary tumor showing eNOS positivity in nearly every tumor cell.

E Strong eNOS positivity is also seen in the majority of C3L5 tumor cells at the site of spontaneous lung metastasis.

F A negative control for eNOS staining of the primary C3L5 tumor, in which the eNOS antibody was replaced with an Ig of the same isotype.

relation between eNOS expression and metastatic ability.

IV. B. NOS expression in C3L5 and C10 tumor lines (Lala, Orucevic and Hum, unpublished)

C3L5 and C10 tumor lines, when transplanted subcutaneously, both give rise to primary tumors which grow to large sizes. However, C10 tumors grow more slowly, are more circumscribed and less invasive. The mean number of spontaneous lung metastases produced 3 weeks after subcutaneous transplantation of 5×10^5 cells in syngeneic mice (n = 15) was 10.7 (median = 8) with C3L5 cells and 1.3 (median = 1) for C10 cells, respectively.

In vitro cultured tumor lines showed positive eNOS staining in nearly 100% of C3L5 cells (Figure 2A), but weak (and heterogeneous) eNOS staining in C10 cells (data not shown). Both cell lines were negative for iNOS. When cultured in the presence of LPS and IFN-γ, C3L5 cells were induced to express iNOS as shown by strong iNOS staining in 25–30% of cells (Figure 2B). C3L5 cells grown *in vivo* maintained strong positivity for eNOS, both in the primary tumors and their spontaneous lung metastasis (Figure 1C and D). These findings were consistent with the notion that eNOS expression by tumor cells provided a selective advantage for invasion and metastasis in this mammary tumor model.

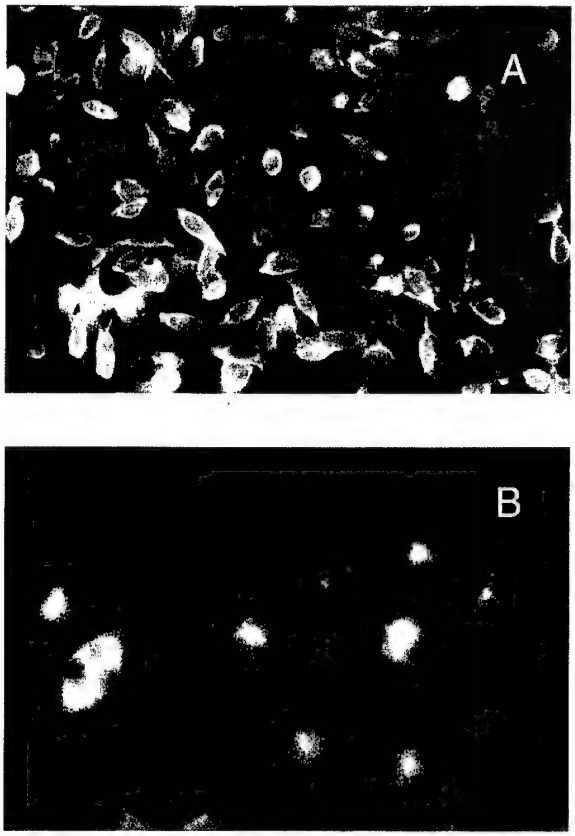

Figure 2. Immunofluorescent staining of cultured C3L5 cells for eNOS (A) under normal culture conditions, and iNOS (B) after 24 h exposure to LPS (10 μg/ml) and IFN-γ (1000 U/ml). Most tumor cells were strongly positive for eNOS (A), and 25–30% of the cells became strongly positive for iNOS (B) following induction with LPS and IFN-γ.

IV.C. Effects of NOS inhibitors (NMMA and L-NAME) on tumor growth and metastasis in C3L5 tumor-bearing mice

Treating C3L5 tumor-bearing mice with two NOS inhibitors [36, 37] provided the first direct evidence of a contributory role of NO in tumor growth and metastasis. NMMA (given repeatedly as two 3 day rounds by the subcutaneous route) as well as L-NAME (administered orally in the drinking water as two 4 day rounds) led to a reduction in the growth of the subcutaneously transplanted primary tumors and their spontaneous lung metastases (Figures 3–6]. The finding of reduced primary tumor growth has since been reproduced with chronic L-NAME therapy administered subcutaneously using osmotic minipumps (Jadeski and Lala, unpublished). Treatment with L-NAME has also been reported to reduce the growth of the primary tumors in a transplanted rat colonic adenocarcinoma model [35], in which the tumor vasculature expressed iNOS. Based on the temporal kinetics of tumor growth after the therapy, the authors suggested that L-NAME reduced blood flow through the tumor vasculature and that native NO was instrumental in promoting the tumor blood flow.

What are the mechanisms underlying NO-mediated promotion of tumor growth and metastasis observed in numerous tumor models? The possibilities, in theory, include: (a) a direct stimulation of tumor cell proliferation; (b) a promotion of tumor

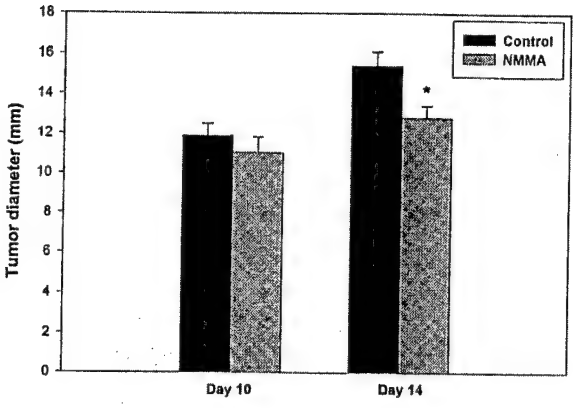

Figure 3. Effects of subcutaneous NMMA therapy for 3 days (20 mg/kg/injection, every 8 hr × 10 injections) on days 10–13 after subcutaneous transplantation of 5×10^5 C3L5 mammary tumor cells, on mean tumor diameter (n = 10–15). There was a significant (* $p < 0.05$) decline in primary tumor size measured on day 14. *(Adapted with kind permission from Orucevic and Lala, Cancer Immunol Immunother, Springer Verlag, 42: 38–46, 1996).*

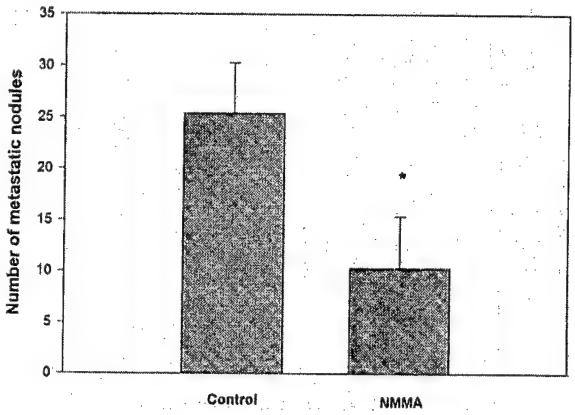

Figure 4. Effects of subcutaneous NMMA therapy given as two 3 day cycles (days 10–13 and 19–22 after subcutaneous C3L5 tumor transplantation, at the same dose rate as in Figure 3), on the number of lung metastatic nodules in mice (n = 9) killed on day 22. The therapy resulted in a significant ($P < 0.05$) reduction in spontaneous lung metastasis. *(Adapted with kind permission from Orucevic and Lala, Cancer Immunol Immunother, Springer Verlag 42: 38–46, 1996).*

cell invasiveness; (c) a promotion of tumor angiogenesis; (d) a promotion of microcirculation in the tumor neovasculature; (e) a suppression of the host anti-tumor defence. Of these, we have gathered evidence in favour of possibilities (b) and (c). The influence of NO in tumor microcirculation is the subject matter of another article (by Fukumura and Jain) in this issue.

V. NO and tumor cell proliferation, survival and apoptosis

Edwards et al. [38] found a discrepancy in tumor cell proliferation *in vitro* and tumor growth *in vivo* after induction of NO with LPS and IFN-γ; *in vitro* induction inhibited tumor cell proliferation, whereas *in vivo* induction promoted tumorigenesis and metastasis. Since C3L5 mammary adenocarcinoma cells expressed active eNOS and produced NO *in vitro*, we tested whether NMMA affected tumor cell proliferation by measuring the uptake of ^{3}H-Thymidine (^{3}HTdR) by C3-L5 cells *in vitro*. NMMA treatment for 24 hours at concentrations capable of blocking NO production *in vitro*, had no influence on ^{3}HTdR uptake when cells were pulsed with ^{3}HTdR during the last 6 hours of 24 hour culture (unpublished data), suggesting that endogenous NO did not directly affect C3L5 tumor cell proliferation.

It has been reported that endogenous or exogenous NO exerts anti-proliferative effects on cells that express functional wild-type p53 tumor suppressor gene [48, 49]. NO stimulates accumulation of the p53 protein in these cells which blocks proliferation by hindering progression of cells through the cell cycle; this may explain cytostatic effects of NO on certain tumor cells: Tumor cells transfected with iNOS were growth-inhibited *in vivo* only when they expressed wild type p53. However, iNOS-expressing tumor cells in which p53 was lost or mutated were resistant to the anti-proliferative effects of endogenous NO, and grew faster *in vivo* than those not expressing iNOS [49].

Apoptosis is another mechanism which can compromise cell survival in the presence of high NO levels. It has been suggested that the NO-dependent component of tumoricidal function of certain immune effector cells is by induction of tumor cell apoptosis [50]. A similar tumoricidal function has also been ascribed to cytokine-activated endothelial cells [51]. Very high levels of endogenous NO production can trigger apoptosis in the NO-producing cell, e.g. in cytokine-activated transformed mu-

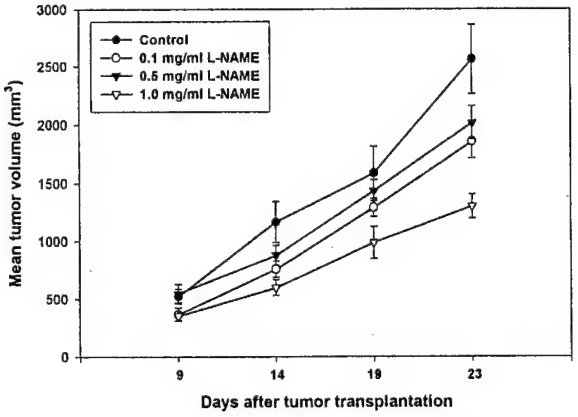

Figure 5. Effects of chronic L-NAME therapy given as two 4 day cycles (days 9–13 and days 19–23) at various concentrations of the drug in the drinking water, on the growth rate of primary tumors following a subcutaneous transplantation of 2.5×10^5 C3L5 tumor cells. Animals drank 3–4 ml water/day (n = 10–20). The data represent means ± SE. There was a dose-dependent decline in tumor growth which was significant at the highest dose (1 mg/ml) throughout the experimental period. *(Adapted with kind permission from Orucevic and Lala, Brit J Cancer 73: 189–196, 1996).*

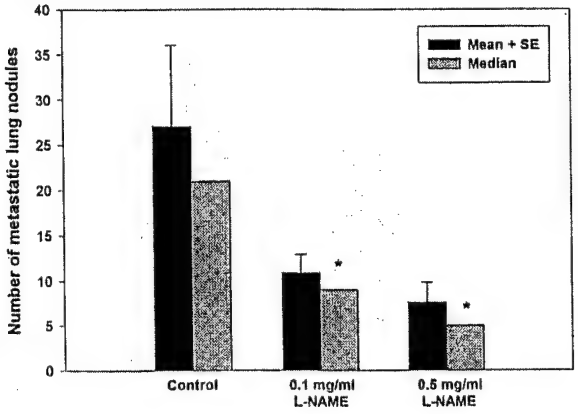

Figure 6. Effects of chronic L-NAME therapy (same dose and schedule as in Figure 5) on the number of spontaneous lung metastatic nodules of C3L5 tumor scored on day 23. There was a significant dose dependent reduction in lung metastasis. *(Adapted with kind permission from Orucevic and Lala, Brit J Cancer 73: 189–196, 1996).*

rine fibroblasts [52]. This phenotype can be detrimental to tumor cell survival. For example, engineered iNOS overexpression in a murine melanoma line abrogated tumorigenic and metastatic ability of these cells because of rapid apoptosis; these cells failed to survive even *in vitro* in the absence of NO-blocking agents [41]. However, tumor cells can vary widely in their susceptibility to NO-mediated apoptosis, from being highly susceptible to totally resistant. Reasons for such variation have not been fully explored. A variation in the genetic makeup can be suggested as one of the reasons. For example, susceptibility is provided by the expression of wild-type p53, and cellular ability to upregulate functional p53 in response to NO, whereas loss or mutation of p53 makes the cells resistant to NO-mediated apoptosis [49, 53, 54]. Furthermore, an overexpression of Bcl2 can protect tumor cells from NO-mediated apoptosis [55, 56].

VI. NO and tumor cell invasiveness: role of tumor-derived NO in the invasiveness of C3L5 cells

This was tested in an *in vitro* matrigel invasion assay [57], in which the invasive ability of tumor cells was determined by measuring the proportion of tumor cells transgressing a matrigel barrier during a 24–72 hour period, after blocking endogenous NO production or inducing additional NO production. Specifically, the effects of adding NOS inhibitors (NMMA, L-NAME in various concentrations) with or without excess L-arginine (which competes with the NOS inhibitors, abolishing their effects), or adding iNOS inducers (LPS in combination with IFN-γ) with or without NOS inhibitors were examined. The results can be summarized as follows. (i) Presence of NMMA or L-NAME reduced the invasion index; a parallel reduction in the level of NO production by the tumor cells measured by the $NO_2^- + NO_3^-$ levels in the medium was observed. (ii) Inclusion of excess L-arginine with NOS inhibitors abrogated the anti-invasive effects of the NOS inhibitors, attesting to the functional NOS specificity of the inhibitors. (iii) Inclusion of LPS and IFN-γ led to a major stimulation of NO production and a concomittant stimulaton of invasiveness. (iv) Inclusion of NOS inhibitors along with LPS and IFN-γ caused only a partial reduction in invasiveness, and LPS and IFN-γ-induced NO production. These findings clearly demonstrated that constitutive NO production by C3L5 tumor cells upregulated their

invasive ability. The invasion-stimulating effects of LPS and IFN-γ could be partially explained by a stimulation of NO production.

What are the mechanisms underlying the invasion-stimulating effects of NO in this tumor model? Earlier studies have shown that NO promotes degradation of articular cartilages by activating matrix metalloproteases (MMP's) in chondrocytes from numerous species [58, 59]. We hypothesized that NO leads to an alteration in the balance between the synthesis of MMP's and the synthesis of their natural inhibitors, i.e., tissue inhibitors of metalloproteases (TIMP's) in tumor cells. To test this hypothesis, the levels of MMP and TIMP mRNA expression were measured in these cells under different experimental conditions. C3L5 cells expressed the 72 kDa type IV collagenase (gelatinase A) and not the 92 kDa species (gelatinase B). They also expressed TIMP-1, TIMP-2 and TIMP-3. Phosphoimage analysis of Northern blots relative to the 18S RNA (loading controls) provided a measure of the mRNA expression under the various experimental conditions. The results are presented in Table 1.

Data presented in Table 1 show that (i) C3L5 cells expressed eNOS but not iNOS mRNA under native conditions, however, iNOS expression was induced in the presence of LPS and IFN-γ. This induction was upregulated by treatment of cells with NMMA, explained by a reduction of the NO-mediated negative feedback on the iNOS gene expression. (ii) NMMA treatment did not affect MMP-2 expression, but upregulated the expression of TIMP-2, and to a minor extent TIMP-3, indicating that the invasion-stimulating effects of endogenous NO are, at least in part, mediated by a downregulation of TIMP-2, and possibly TIMP-3. (iii) LPS and IFN-γ treatment upregulated MMP-2 and down-regulated TIMP-3, explaining the invasion-stimulating effects. Addition of NMMA to LPS and IFN-γ restrained the MMP-2 expression to normal level and only partially restored TIMP-3 expression, thus explaining the incomplete abrogation of LPS and IFN-γ stimulation of invasiveness with NOS inhibitors. These results indicated that LPS and IFN-γ-mediated stimulation of invasiveness is only partially explained by increased NO production, and that NO at higher levels can upregulate MMP-2 and downregulate TIMP-3. Indeed, we have observed that exposure of C3L5 cells to S-Nitroso-N-Acetyl-D, L-penicillamine (SNAP) (an NO donor) downregulates TIMP-3 mRNA (data not shown). Assuming that gene expression was directly related to the secretion of protein products, these results suggest that NO-mediated stimulation of invasiveness is due to an alteration in the balance between productions of the MMP's and TIMP's. Furthermore, NO has been shown to upregulate urokinase type plasminogen activator (uPA) in endothelial cells of post capillary venules during the process of NO-mediated stimulaton of angiogenesis [60]. Since uPA converts plasminogen to plasmin, which can activate numerous MMP's, this may represent another pathway of NO-mediated stimulation of matrix degradation.

Further studies of the relationship of NO to tumor cell invasiveness are needed using different tumor models. In K1735 murine melanoma cells, engineered overexpression of iNOS, leading to de-

Table 1. Image analysis* of Northern blots of mRNA expression in C3L5 cells

Transcript	Control	IFN-γ and LPS	IFN-γ and LPS + NMMA	NMMA
eNOS (4.5 kb)	1.0	1.0	1.0	1.0
iNOS (4.8 kb)	–	+	++	–
MMP-9 (2.5 kb)	–	–	–	–
MMP-2 (3.1 kb)	1.0	1.7	0.9	1.0
TIMP-1 (3.1 kb)	1.0	0.9	1.0	1.0
TIMP-2 (3.5 kb & 1.0 kb)	1.0	0.8	0.6	1.3
TIMP-3	1.0	0.3	0.6	1.2

* Standardized with 18S RNA used as a loading control. A positive expression in control cells is normalized to 1.0.
IFN-γ = 500 u/ml; LPS = 10 μg/ml; NMMA = 1 mM.

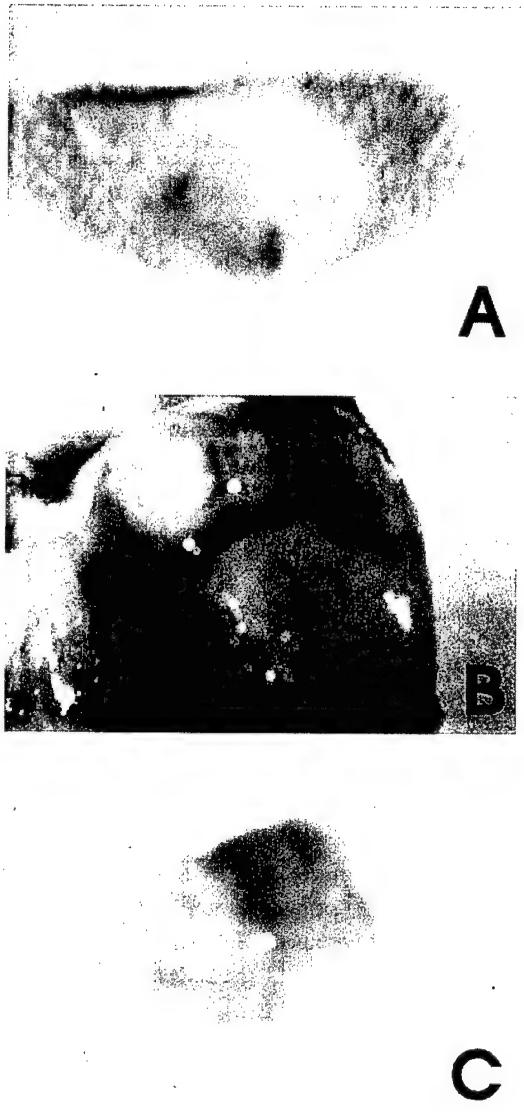

Figure 7. Gross morphology of matrigel implants in (A) matrigel alone implanted in D-NAME treated mice; (B) matrigel inclusive of tumor cells in D-NAME treated mice, and (C) matrigel inclusive of tumor cells in L-NAME treated mice. Note that implant A is mostly avascular, and implant B has grown in size and highly vascular, whereas implant C is smaller in size and less vascular in comparison with B.

creased tumor cell survival and tumorigenicity [41], has been reported to be associated with a down regulation of MMP-2 [61] owing to a downregulaton of MMP-2 promoter activity. It is thus possible that very high levels of endogenous NO may compromise invasive function of certain tumor cells, which are susceptible to NO-mediated cytotoxicity.

VII. NO and angiogenesis

VII. A. Roles of NO in angiogenesis under physiological conditions and during wound healing

Regulatory roles of NO in angiogenesis remain somewhat controversial. Pipili-Synetos *et al.* [62] suggested that NO is an endogenous inhibitor of angiogenesis. Using an angiogenesis assay which scores the number of blood vessels in a defined area of chick chorio-allantoic membrane (CAM assay), as well as tube formation by endothelial cells on matrigel, they found that Na-nitroprusside (an NO-donor) reduced and NOS inhibitors promoted basal angiogenesis. In contrast, Konturek *et al.* [63] found that inhibition of NO synthesis delayed healing of chronic gastric ulcers induced by acetic acid, by reducing local blood flow and angiogenesis at the periphery of the ulcers. Similarly, using both an *in vivo* angiogenesis assay with rabbit cornea and an *in vitro* assay which measures the growth and migration of capillary endothelial cells, Ziche *et al.* [64] showed that vasoactive substances such as substance P, or prostaglandin E $(PGE)_1$ stimulated angiogenesis in an NO-dependent manner, since it was blocked with NOS inhibitors NMMA, N^G-nitro-L-arginine (L-NNA) and L-NAME. NO donors such as Na-nitroprusside and glycerol trinitrate also stimulated endothelial cell migration. These authors have reported that one of the final pathways of NO-mediated angiogenesis is by upregulation of basic fibroblast derived growth factor (bFGF) in post capillary venule endothelial cells [60]. Angiogenic activity of human monocytes [65] and mitogenic activity of VEGF on coronary venular endothelium [66] have also been shown to be NO dependent. Indeed, NO was shown to be a downstream mediator of VEGF but not bFGF-induced angiogenesis [87].

It is possible that the conflicting data cited above on the role of NO in angiogenesis is due to the differences in levels of NO and NO scavengers in the microenvironment. Very high levels of NO may be

cytostatic or apoptosis-inducing for endothelial cells, whereas low to moderate levels of NO may promote endothelial cell migration, invasiveness and differentiation, either directly or by induction of angiogenic factors such as bFGF. A continuous remodelling of blood vessels, requiring highly localized endothelial cell death may be facilitated by high local endogenous NO levels (hot spots) in an embryonic tissue such as the CAM, which was utilized in the angiogenesis assay by Pipili-Syntos *et al.* [62]. This may account for the anti-angiogenic role of NO observed using this model, as opposed to the angiogenic role of NO in other models in which the basal level of NO may be low or negligible.

VII. B. Role of NO in tumor angiogenesis

Buttery *et al.* [34] suggested that iNOS expression by endothelial cells of the neovasculature of many experimental tumors promoted angiogenesis as well as blood flow in the vasculature, and thus sustained tumor growth. This suggestion has been reinforced by other investigators [67], and validated by Jenkins *et al.* [39] showing that the increased *in vivo* growth resulting from iNOS transfection of a human colon cancer cell line was associated with increased vascularity of the transplants in nude mice.

Table 2. NO and tumor biology: variables

Source of NO in the tumor:
Tumor cells (eNOS, nNOS, iNOS)
Tumor endothelium (eNOS, iNOS)
Tumor stroma, macrophages (iNOS)
Level of NO production by the tumor:
eNOS, nNOS: low to moderate
iNOS: moderate to high (high in an inductive environment)
Role of NO in tumor biology:
Genetic makeup of the tumor, for example:
wt $p53^{+}$: cytostasis, apoptosis (especially with high NO levels)
p53 null, mutant, inactive: resists NO mediated injury, and uses NO for tumor progression
Levels of NO:
High: cytostasis, apoptosis (in a susceptible genetic make up)
Low to moderate: promotion of tumor progression by increased angiogenesis, tumor microcirculation and tumor cell invasiveness

The latter findings have since been confirmed with other tumor cell lines transfected with iNOS, when the tumor cell lines had lost or mutated p53 [49].

Angiogenic role of NO in the C3L5 mammary tumor model (Jadeski, Hum, Orucevic and Lala, unpublished)

We gathered two types of evidence for the angiogenic role of NO in the C3L5 tumor model. (i) Tumors of the same age in mice subjected to NMMA therapy were compared with those in animals treated with vehicle alone for the incidence of blood vessels per unit area of the section of tumor tissue (Orucevic and Lala, unpublished). Tumors in NMMA-treated mice exhibited a significant reduction in the incidence of blood vessels. (ii) We designed a tumor angiogenesis assay by adapting the protocol of Kibbey et al. [68]. Rehydrated matrigel, which is liquid at 4 °C and solidifies at body temperature, was implanted subcutaneously in mice. The matrigel pellet stimulates the ingrowth of new blood vessels from the periphery of the implant, possibly because of the presence of angiogenic factors in conventional matrigel and by Kibbey *et al.* [68]. In our experiments, we replaced the conventional matrigel with growth factor-reduced matrigel which, on its own, stimulated little or no angiogenesis (Figure 7A) in C3H/HeJ female mice. However, inclusion of an appropriate number of C3L5 tumor cells in the matrigel was highly angiogenic. When animals were placed on chronic subcutaneous L-NAME therapy via osmotic minipumps shortly following the implantation, both the vascularity and the size of the tumor cell-inclusive implants declined (gross appearance shown in Figure 7C) as compared to the implants in control mice receiving vehicle alone or D-NAME therapy (Figure 7B). None of the therapies had any influence on the vascularity of the tumor cell-exclusive implants. These preliminary data suggest that an inhibition of NO production led to a reduced angiogenic ability of the tumor cells in the implants. Whether the decline in the growth of tumor cells was secondary to the angioreductive effects alone remains undetermined.

VIII. NO and host immune responses

A number of reports suggest opposing roles of NO in tumor immunity. Activated murine macrophages synthesize NO [69], which may partly mediate their cytotoxic activity against tumor cells [17, 50, 59], bacteria [16] and parasites [71]. Mills *et al.* [72] reported that ascites tumor growth in the mouse peritoneal cavity was associated with a reduced NO production by intratumor macrophages. Similarly, it has been reported that *in vitro* tumoricidal function of activated natural killer (NK) cells depends partly on their NO synthesizing ability [73–75]. In contrast, NO overproduction by rodent macrophages has been shown to suppress proliferation of T lymphocytes in response to antigens or mitogens [76, 77], and thus may hinder anti-tumor immune responses of T cells. Indeed, excessive NO production has been implicated in tumor-induced immunosuppression in rats [78]. We tested whether a potentiation of interleukin-2 (IL-2)-induced regression of C3L5 mammary tumors [37] resulting from L-NAME therapy can be explained, at least in part, by a potentiation of lymphokine activated killer (LAK) cell activation. We found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2-induced generation of anti-tumor cytotoxicity of splenocytes in healthy as well as mammary adenocarcinoma-bearing mice; there was a parallel drop in IL-2-induced NO production *in vivo* and *in vitro* [79]. These results revealed that the IL-2-induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition. In our hands, NO inhibitors added during the cytotoxicity assays had no detrimental effect on LAK cell mediated anti-tumor cytotoxicity. In summary, NO appears to be an important bioactive component of the cytotoxic pathways of anti-tumor effector cells, in particular macrophages. However, sustained NO release in the immune microenvironment is also detrimental to effector cell activation pathways, and thus suppress their anti-tumor function.

IX. Conclusions and suggestions

The above evidence suggest that NO may play opposing roles in tumor growth and metastasis. The precise role of NO produced by tumor cells or host cells in the tumor microenvironment may depend on two variables: (a) the level of NO production and (b) the genetic makeup of the tumor cells. Very high levels of NO can be detrimental to the survival of certain tumor cells as well as host cells because of NO-mediated cellular injury, cytostasis and apoptosis. Since high NO-producing tumor cell clones would likely delete themselves *in vivo*, it is reasonable to postulate that in most well established spontaneous tumors exhibiting a clonal heterogeneity, those producing low to moderate levels of NO or those capable of resisting NO-mediated cytostasis and apoptosis will survive and propagate. For these cells, tumor or host-derived NO may have a facilitating role for tumor progression by virtue of NO-mediated stimulation of invasiveness, angiogenesis and microcirculation within the tumor. These events may assume greater significance within the hypoxic regions of a tumor. In a solid tumor, hypoxia is known to provide a stimulus for angiogenesis by various pathways [80]. One of the mechanisms is the induction of vascular endothelial growth factor (VEGF) which has a hypoxia-responsive element in the promoter region of the gene. Hypoxia can also induce iNOS in a similar manner by activating the iNOS promoter via a hypoxia-responsive element [81]. The hypoxia-induced NO production may provide additional angiogenic and invasion-stimulating signals within a solid tumor.

The genetic makeup of tumor cells that may dictate susceptibility of resistance to NO-mediated injury remains to be completely investigated. One of the possible genetic determinants is the functional status of the p53 tumor suppressor gene. It has been shown that iNOS-transfected tumor cells expressing wild type functional p53 are vulnerable to NO-mediated cytostasis, because of an accumulation of p53 protein induced by endogenous NO [48, 49]. However, p53 accumulation eventually leads to a transcriptional transrepression of iNOS and thus inproves cellular survival [48]. Tumor cells in which p53 gene is deleted or mutated (causing a loss of

normal p53 function), on the other hand, can withstand NO-mediated cytostasis or apoptosis [49, 53, 54]. In addition, in the presence of endogenous NOS, p53-deficient or mutant tumor cells exhibit faster tumor growth and vascularity, when transplanted *in vivo* [49]. These observations led to the hypothesis that interaction of NO with p53 provides one mechanism of clonal selection of p53 mutant or p53 null cells which can utilize NO to their advantage for tumor progression *in vivo* [49, 82]. Since p53 mutation occurs in nearly half of all human cancers [83], this hypothesis predicts that NO would facilitate progression in a large proportion of human cancers. It remains to be seen whether a p53 dependent role is universal to all tumors and whether other tumor suppressor genes, e.g., Rb may interact with NO in a similar manner. The picture can be complicated further by other (nongenetic) mechanisms of protection from NO-mediated injury. For example, certain cells show acquired resistance to NO-mediated injury after prior exposure to NO [84, 85]. Nevertheless, availability of reliable genetic markers which can predict the specific role of NO in tumor biology will be highly valuable in determining the applicability of NOS inhibitors in treating specific tumors.

Table 2 presents a schema of the variables that may dictate the role of NO in tumor biology.

X. Key unanswered questions

1. Can the opposing roles of NO (on tumor progression vs. tumor regression) be explained by (a) the level of NO production, eg. high vs. moderate to low, (b) the ability of the tumor cell type to resist NO-mediated injury, or (c) both? Further experimentation using different tumor systems is needed to answer these questions. In particular, genetic determinants which may allow tumor cells to resist NO-mediated injury and exploit NO to their advantage deserve full exploration.
2. How universal is the phenomenon of NO-mediated promotion of tumor angiogenesis? This area needs further investigation using multiple tumor models with different NO producing abilities *in vivo*.
3. What are the effects of disrupting or down-regulating the eNOS gene in the high eNOS-expressing and highly metastatic C3L5 mammary tumor cells on their invasive, angiogenic and metastatic abilities? Conversely, what are the results of up-regulating eNOS in the low eNOS expressing, poorly metastatic C10 mammary tumor cells?

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Role of nitric oxide in IL-2 therapy-induced capillary leak syndrome

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Key words: interleukin-2, capillary leak syndrome, nitric oxide, nitric oxide synthase inhibitors, NMMA, L-NAME, murine mammary adenocarcinoma

Abstract

Nitric oxide (NO) is a potent short-lived and short range bioactive molecule, which plays a key role in physiological and pathological processes including inflammation and cancer. Detrimental effects of excessive NO production during septic shock have been well recognized. We tested the hypothesis that 'capillary leak syndrome' following systemic interleukin-2 (IL-2) therapy resulted from a cascade of events leading to the induction of NO which, directly or indirectly, injured capillaries and caused fluid leakage. Our results provided the first direct evidence that the induction of active NO synthase (NOS) leading to the overproduction of NO is instrumental in IL-2-induced capillary leakage in mice and that successful blocking of this overproduction with chronic oral administration of NOS inhibitors can mitigate this leakage without interfering with the beneficial antitumor effects of IL-2 therapy. NO blocking agents can, in fact, improve IL-2-induced antitumor effector cell activation, as well as tumor regression. In our studies, NO blocking agents alone reduced the growth and metastasis of a murine mammary carcinoma, at least in part, by mitigating the invasion and angiogenesis-stimulating role of tumor-derived NO. Thus, NOS inhibitors may be useful in treating certain tumors and serve as valuable adjuncts to systemic IL-2 based immunotherapy of cancer and infectious diseases.

Introduction

During the last decade, use of systemic interleukin-2 (IL-2) became a major focus of interest in cancer immunotherapy because of IL-2 dependence of all anti-tumor effector cells i.e. T cells [1], natural killer (NK) cells [2] and macrophages [3, 4]. The success of high dose IL-2 therapy in metastatic murine cancers [5] soon led to human trials with IL-2 alone or in combination with *ex vivo* generated lymphokine-activated killer (LAK) cells [6–10], or in combination with chronic indomethacin therapy [11, 12] resulting in modest and variable success in renal cell carcinomas and melanomas.

Wide spread clinical use of systemic IL-2 based therapy, has been limited by a major side effect known as 'capillary leak syndrome'. It is characterized by retention of extravascular fluid, severe hypotension, and multiple organ system dysfunction [13, 14], often requiring cessation of IL-2 therapy. This syndrome has been documented in numerous species: humans [13, 15, 16], mice [17, 18], sheep [19–21] and rats [22].

Reported pathophysiological mechanisms underlying this syndrome include damage of endothelial cells by LAK cells [23, 24] or NK cells responding to IL-2 [25] or certain IL-2 induced cytokines e.g. interferon (IFN) γ [26] and tumor necrosis factor (TNF) α [27]. Injury to endothelial cells mediated by these cytokines has been recently linked with nitric oxide (NO) production [28, 29], because it was prevented with dexamethasone and inhibitors of NO synthesis. Severe hypotension observed during IL-2 therapy has also been recently attribut-

ed to NO production [30, 31]. NO is synthesized by many mammalian cells from the amino acid L-arginine, with the help of a family of enzymes called NO synthases (NOS) [32, 33]. It is a short lived biological mediator of many physiological functions. However, sustained overproduction of NO resulting from the induction of the inducible isoform of NOS (iNOS) may have pathological consequences including capillary damage because of cytotoxic action on endothelial cells [28, 29]. Vasodilation [34] and systemic hypotension due to NO production can indirectly cause pulmonary hypertension, and the increased pulmonary capillary pressure [13] can lead to fluid leakage in the lungs. Thus, NO may have a major role in the pathogenesis of IL-2 induced capillary leakage. In this paper, we shall briefly review the IL-2 based cancer therapies and possible pathways of IL-2 therapy induced capillary leakage. Since IL-2 therapy induces production of LAK cells [16, 35], IFNγ [36], TNFα [15, 37], and NO [30, 31], we shall discuss the independent as well as interdependent roles of these multiple factors. We shall show that NO overproduction occurs at the later part of a cascade responsible for this syndrome and that appropriate administration of NOS inhibitors can not only overcome the syndrome but also improve antitumor effects of IL-2 therapy.

Systemic IL-2 in tumor immunotherapy

A. Biology of IL-2: Tumor therapy with IL-2 as a single agent

T cell growth factor (later named as IL-2) was initially identified in the supernatant of phytohemagglutinin-stimulated normal human lymphocytes that supported the growth of T cells in culture of normal human bone marrow [38]. IL-2 has since been characterized as a 133 amino acid polypeptide of 15,500 daltons [39], in humans it is encoded by a single gene [40] on chromosome 4. Recombinant IL-2 has been obtained by inserting the IL-2 gene from cultured leukemic cells [41] or from normal peripheral blood lymphocytes [42] in *Escherichia coli*. This form of recombinant IL-2, although non-glycosylated, has biological activity *in vitro* and *in vivo* identical to that of native IL-2.

The structure of the IL-2 receptor consists of 3 peptide chains (α, β and γ); the genes encoding these chains have been cloned and characterized [43, 44]. The α chain alone provides a receptor of low affinity. Intermediate and high-affinity receptors are produced by β/γ heterodimer and α/β/γ heterotrimer, respectively, in which the β chain is critical for signal transduction [45]. IL-2 receptor expression has been variably found on the surface of T cells, NK cells [2, 46], macrophages [3, 4, 47], oligodendroglial cells [48], epidermal Langerhans cells [49], B cells [50], and certain tumor cells lines derived from melanomas and squamous cell carcinomas of the head and neck [51].

The tumoricidal potential of all immune effector cells including T cells [1], NK cells [2, 52, 53], and macrophages [3, 4] can be stimulated with IL-2. Lymphocytes cultured in the presence of high dose IL-2 lead to the activation of NK cells and T cells, providing a heterogenous population of cytotoxic cells with a broad spectrum of antitumor cytotoxicity, known as LAK cells which are capable of killing syngeneic as well as allogenic tumor cells [54]. This knowledge provided the impetus for systemic IL-2 therapy of cancer.

Rosenberg *et al.* [5] were the first to show that systemic administration of IL-2 resulted in regression of pulmonary metastasis in mice by activation of LAK cells *in vivo*. These findings led to the application of IL-2 therapy in human cancers, revealing that highest tumor regression occurred in melanomas and renal cell carcinomas [16, 55, 56].

B. Systemic IL-2 in combination with LAK cells in tumor therapy

Rosenberg's group observed that a combination of systemic IL-2 therapy with infusion of LAK cells generated *in vitro* had significantly higher antitumor activity in mice than IL-2 therapy alone [57, 58]. Intravenous IL-2 therapy in combination with LAK cells was then applied to treat human patients with solid tumors. Autologous lymphocytes were obtained from cancer patients by repeated leukapheresis, cultured in the presence of IL-2 to generate LAK cells, and reinfused into the patients together with IL-2 [16]. This treatment resulted in the regression of tumors in some patients for whom no

other effective therapy was available [35, 59]. However, it was soon apparent that the therapeutic benefit derived from this combination therapy was not greater than that from IL-2 therapy alone [7].

C. IL-2 in combination with tumor infiltrating lymphocytes in tumor therapy

Lymphocyte-trafficking studies with radiolabeled LAK cells generated from blood or splenic lymphocytes showed that LAK cells did not localize at tumor metastatic sites but were trapped in the lungs and later in the liver. However, lymphocytes retrieved from the tumor and expanded with IL-2 showed some selectivity for migration to the tumor metastatic site after infusion *in vivo* [60]. These 'tumor infiltrating lymphocytes' (TIL) were expanded *in vitro* [61] for adoptive transfer. When TILs were infused along with IL-2 in patients with melanoma or renal cell carcinoma, responses were higher relative to IL-2 treatment alone or IL-2 combined with LAK cells [61–63].

D. IL-2 in combination with chronic indomethacin therapy in tumor treatment

Lala *et al.* [64] observed that natural killer cells were progressively inactivated in the tumor-bearing host with increasing tumor burden. This inactivation was caused by a high level of prostaglandin E_2 (PGE_2) produced by host macrophages [65] as well as certain tumor cells [66]. PGE_2 has been shown to suppress lymphocyte proliferation [67] and activation of all antitumor killer cell lineages [4]. These effects, at least in part, explained the promotion of metastatic ability of tumors by PGE_2 [68]. The PGE_2-mediated inactivation of effector cells was attributed to inhibition of IL-2 production [69] and a down regulation of IL-2 receptors on the surface of all killer cell lineages [70].

Based on these findings, Lala's group started an immunotherapy protocol combining systemic IL-2 with chronic oral administration of indomethacin [71], a drug that inhibits prostaglandin production [67]. Chronic indomethacin therapy had antitumor and antimetastatic effects [72, 73], and substantially restored natural killer cell function [73] in murine tumor models. However, this therapy alone was unable to eradicate advanced metastases [71], possibly because of inadequate IL-2 production *in vivo*. Chronic indomethacin therapy (given in the drinking water) when combined with systemic injections of IL-2 resulted in permanent regression of B16F10 melanoma metastases in the lungs of a large proportion of animals [74]. Reactivation of AGM-1 + and Thy-1 +/– killer lymphocytes *in situ* accounted for the therapeutic benefit, since depletion of these cells *in vivo* abrogated the therapeutic effects. Similar eradication of metastases was also achieved in C3-L5 mammary adenocarcinomas [75] and human melanomas grown in nude mice [76]. This combination therapy was then applied in a phase 2 human trial of advanced melanoma and renal cell carcinoma patients resulting in good objective responses [11, 12], comparable with the higher ranges in the success rates reported in the other IL-2 based therapy trials [77]. Interestingly, the toxicity was manageable in a general oncology ward without the need for vasopressor agents often used in other IL-2 trials [77], and some melanoma patients responded to indomethacin therapy alone [78].

Capillary leak syndrome due to systemic IL-2 therapy

Initially, it was believed that the efficacy of IL-2 in the therapy of cancer improved as a function of the IL-2 dose administered [56, 59]. Although true for animal models, this association was very weak in a controlled study in renal cell carcinoma patients, receiving high or low-dose of intravenous IL-2 [14], and undetectable in a study in renal cell carcinoma patients using indomethacin in combination with IL-2 [11, 12, 79]. However, dose-related toxicity was observed in most trials and still remains a major obstacle to systemic IL-2 based therapy. Capillary leak syndrome is the most serious side effect of moderate to high doses of IL-2 observed in many species [13, 15, 18, 20–22]. There is an increase in microvascular permeability causing marked accumulation of extravascular fluid in all organ systems and hypotension, often requiring treatment with intravenous fluids and vasopressor agents [81]. Retention of extravascular fluid results in rapid weight gain of up to 20%, manifested by peripheral edema, pleural effu-

sion and ascites [13, 14]. Occasionally, life threatening pulmonary edema, respiratory or cardiac failure, and neurological abnormalities resulting in coma (due to edema of the brain) may develop during IL-2 therapy, requiring cessation of the therapy [13, 14]. Interestingly, symptoms of capillary leakage begin to reverse within 24 h of cessation of IL-2 therapy and usually completely disappear within a few days [14]. Capillary leak syndrome has been observed with IL-2 therapy alone and IL-2 therapy in combination with LAK cells or TIL. A less severe form of the syndrome has also been noted with IL-2 therapy in combination with indomethacin therapy.

Several studies have combined IL-2 with other agents to ameliorate the capillary leakage. However, the added drugs also blocked or reduced the beneficial antitumor effects of IL-2. Corticosteroids [17], which suppress inflammatory responses and induction of NO [82], and asialo-GM-1 antibody, which depletes LAK cells [18], both fall in this category. Puri *et al.* [83] reported that IL-1α reduced IL-2-induced capillary leakage but did not improve animal survival. Welbourn *et al.* [84] reported that certain cyclopeptides (e.g. antamanide and phalloidin), reduced IL-2-induced edema in the rat, presumably by causing cytoskeletal changes in neutrophils with consequent suppression of endothelial injury by thromboxane B_2. Influence of these agents on the antitumor effect of IL-2 remains unknown. Further studies were therefore required to identify substances that can ameliorate capillary leakage without compromising the anti-tumor effects of IL-2.

Based on the observations that systemic IL-2 therapy in combination with chronic indomethacin therapy in advanced melanoma and renal cell carcinoma patients [11, 12] was associated with less severe IL-2 toxicity than reported in the case of other IL-2-trials, we tested in a mouse model whether PGE_2 played any role in the IL-2-induced capillary leakage [85, 86]. Our results revealed that addition of chronic indomethacin treatment markedly improved the antitumor effects of IL-2 therapy, but was unable to ameliorate the IL-2-therapy – induced capillary leakage.

A. Possible mechanisms of IL-2 induced capillary leak syndrome

At least one of two conditions must be satisfied to cause capillary leakage: capillary endothelium must be damaged or the capillary pressure increased. Five mechanisms have been proposed by which IL-2 therapy can induce capillary leakage.

(i). IL-2 induces LAK cells to adhere to and later damage endothelial cells.

(ii). IL-2 induces NK cells to adhere to and damage endothelial cells.

(iii). Endothelial cells are damaged by TNF-α produced by IL-2-activated leukocytes.

(iv). Changes in endothelial cell architecture are caused by IFNγ produced by IL-2-activated leukocytes.

(v). IL-2 directly or indirectly induces NO production which is toxic for endothelial cells. In addition, NO, because of its vasodilatory role, leads to systemic hypotension which indirectly causes pulmonary hypertension resulting in an increase in pulmonary capillary pressure and thus pulmonary edema.

A.1. LAK cells and capillary leak syndrome. LAK cells have been shown to adhere to endothelial cells and cause their lysis *in vitro* [23, 24, 87]. Kotasek *et al.* [23] proposed that the dense granules secreted by LAK cells, which contain serine esterase I (an enzyme with high proteolytic and cytolytic activity, caused breaches in the endothelial cell membranes. Observations on cultured endothelial cells led Savion *et al.* [88] to propose that LAK cells migrated through and ruptured endothelial cell tight junctions. Once they reached the basement membrane and the subendothelial matrix, LAK cells would degrade the matrix by producing matrix-degrading enzymes. These events, with or without endothelial cell lysis, would result in capillary leakage. The hypothesis of LAK cell mediated capillary injury is substantiated by the findings that LAK cell depletion *in vivo* by treatment with asialo-GM-1 antibody in mice ameliorated IL-2 therapy induced capillary leakage [18]. However, this treatment also abrogated antitumor effects of IL-2.

A.2. NK cells and capillary leak syndrome. Aronson

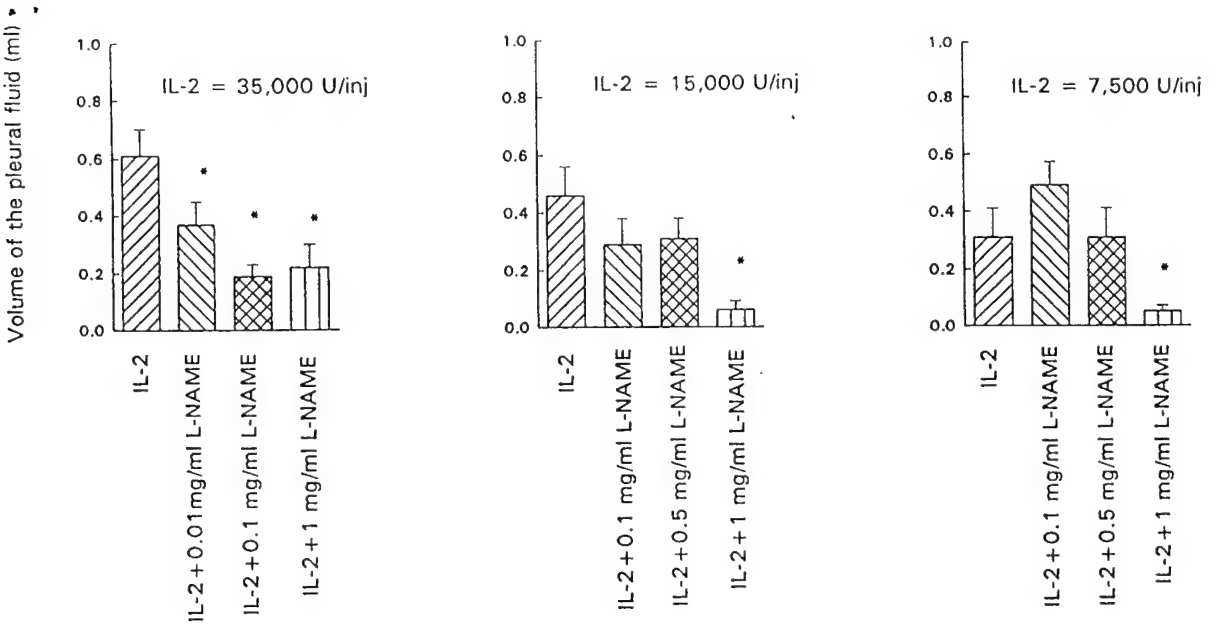


Figure 1. Pleural effusion after IL-2 and L-NAME therapy in healthy mice. Data represent mean ± SE (n = 5). * indicates significant difference from IL-2 treatment ($p < 0.05$).
L-NAME (0.01, 0.1 or 1 mg/ml of drinking water) significantly ($p < 0.05$) reduced IL-2 (35,000 U/inj i.p., every 8 h, 10 inj. total) induced pleural effusion. Significant reduction ($p < 0.05$) of pleural effusion induced by lower IL-2 dose (15,000 U/inj or 7,500 U/inj) was noticed only with high L-NAME dose (1 mg/ml of drinking water). Neither control (untreated) nor L-NAME alone treated mice showed any pleural effusion (data not shown). *Reproduced with kind permission from Orucevic and Lala, J Immunother., 18: 210–220 1996 Lipincott Raven Publishers.*

et al. [25] showed that IL-2 can induce NK cells to adhere to human endothelial cells in culture. These authors implied that vascular leakage induced by IL-2 resulted from NK cell mediated endothelial cell injury. However, there has been no direct evidence of NK cells causing endothelial cell damage *in vivo*.

A.3. TNFα and capillary leak syndrome. IL-2 therapy activates leukocytes (monocyte-macrophage in particular) to produce TNFα [15, 37]. Several authors have reported controversial findings about the ability of TNFα to damage endothelial cells. Collins *et al.* [89] reported that TNFα activated human endothelial cells to express class 1 HLA antigen, suggesting that TNFα made them prone to cytolytic T lymphocyte mediated injury. Kahaleh *et al.* [27] showed that TNFα inhibited endothelial cell growth in culture, and at high concentrations, induced endothelial cell lysis.

In 1990, Doukas and Pober [90] reported that TNFα led to endothelial cell 'activation', which was enhanced further by IFNγ. 'Activation' was indicated by appearance of new morphologic, antigenic and functional characteristics of endothelial cells. Increases in specific endothelial cell surface molecules like ELAM-1 (endothelial leukocyte adhesion molecule 1) or ICAM (intercellular cell adhesion molecule) were observed by these authors after stimulation by TNFα and IFNγ. IL-6 production in response to TNFα was observed by Leewenberg *et al.* [91]. Endothelial cell activation and increased adhesiveness for leukocytes were implied to play a role in increased capillary permeability.

In contrast, Mier *et al.* [92], reported that TNFα and IFNγ activated endothelial cells and increased the binding of CD16 + lymphocytes to endothelial cells in culture, but that the lymphocyte binding was not responsible for increased capillary permeability. In fact, these authors reported that TNFα and IFNγ protected endothelial cells from LAK cell-mediated injury.

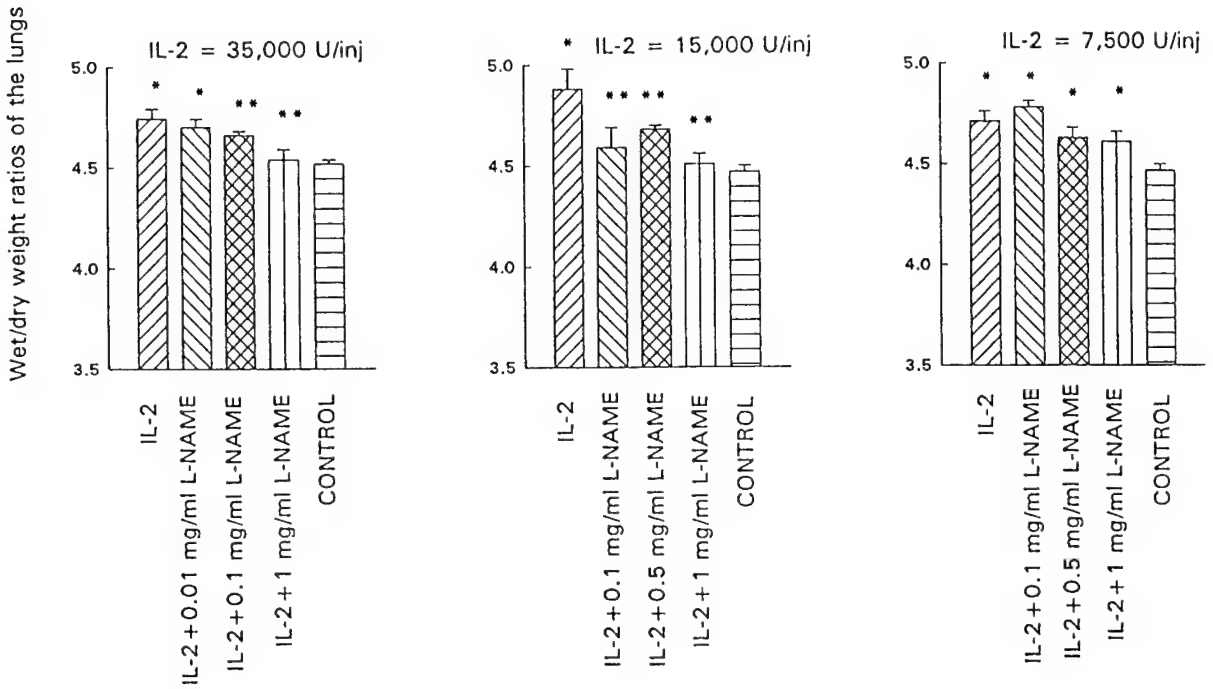


Figure 2. Water content of the lungs after IL-2 and L-NAME therapy in healthy mice. Data represent mean ± SE (n = 5). * indicates significant difference from control ($p < 0.05$). ** indicates significant difference from IL-2 treatment ($p < 0.05$). IL-2 (35,000 U/inj or 15,000 U/inj i.p., every 8 h, 10 inj total) induced pulmonary edema was significantly ($p < 0.05$) reduced with addition of L-NAME (0.1 mg/ml or more) in a dose dependent manner, being abolished at a dose of 1 mg/ml. Low dose of IL-2 (7,500 U/inj) also induced pulmonary edema, but addition of L-NAME did not have any significant effect. *Reproduced with kind permission from Orucevic and Lala J Immunother., 18: 210–220, 1996 Lupincott Raven Publishers.*

A.4. Changes of endothelial architecture induced by IFNγ. IFNγ appears in the blood of cancer patients within 6 hours after administration of IL-2 [15]. Cytotoxic activity of IFNγ is well known, but its possible role in IL-2 induced capillary leak syndrome remains obscure. Montesano *et al.* [26] showed that certain lymphokines could alter human endothelial cell architecture *in vitro*. IL-2 had no effect, and IFNγ had only a marginal effect. Combination of IL-1 and IFNγ completely changed the appearance of endothelial cells. They became elongated with many 'dendrite like' processes, and there were changes in cytoskeletal structure.

A causal relationship between changes in endothelial cell morphology induced by these lymphokines *in vitro* and in the capillary leak syndrome *in vivo* remains to be established. In fact, Puri *et al.* [83] reported that administration of recombinant IL-1 *in vivo* reduced IL-2 induced vascular leakage in the lungs of mice. These authors could not explain the failure of IL-1 to increase survival of mice treated with IL-2 or with IL-2 and IFNγ.

A.5. NO and capillary leak syndrome. Based on the findings that NO can be produced by activated macrophages after treatment with endotoxin, IFNγ or certain other cytokines [93, 94], Kilbourn and Belloni [95] investigated the effects of IFNγ, TNFα, IL-1, IL-2 and endotoxin on the production of NO by endothelial cells. They showed that culture of murine brain endothelial cells produced NO in response to various combinations of cytokines. They speculated that endothelium-derived NO played a role in the development of hypotension in patients treated with IL-2 or TNFα. In support of this hypothesis, Kilbourn *et al.* showed that therapy with N^G-Methyl-L-Arginine (NMMA, an inhibitor of NO synthesis) protected dogs against hypotension induced by TNFα and endotoxin [96, 97], as well as IL-2 [80].

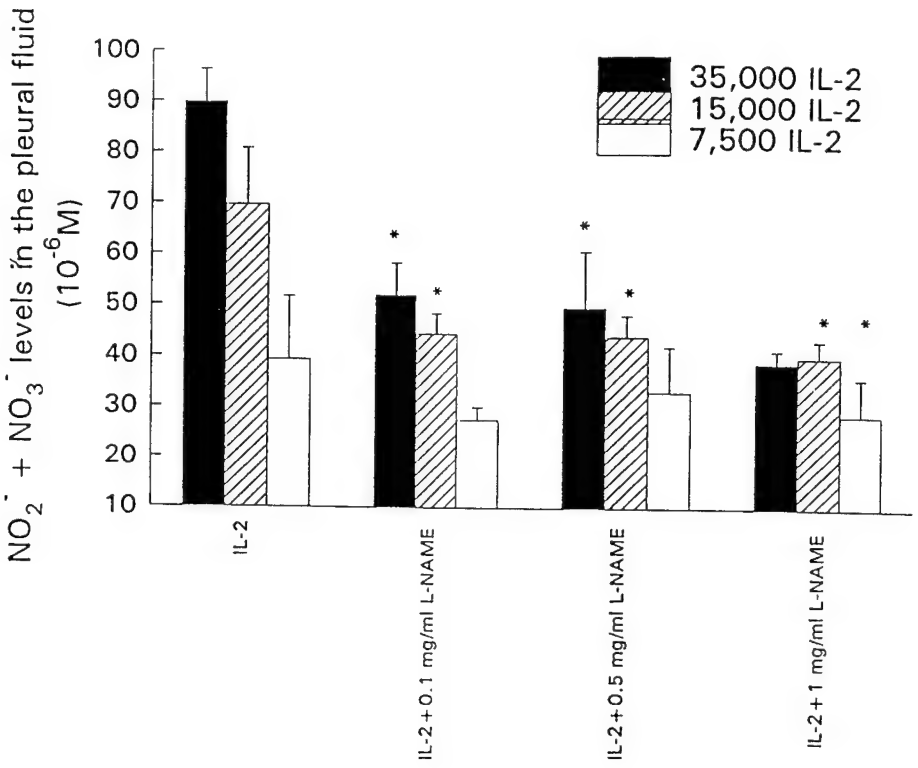
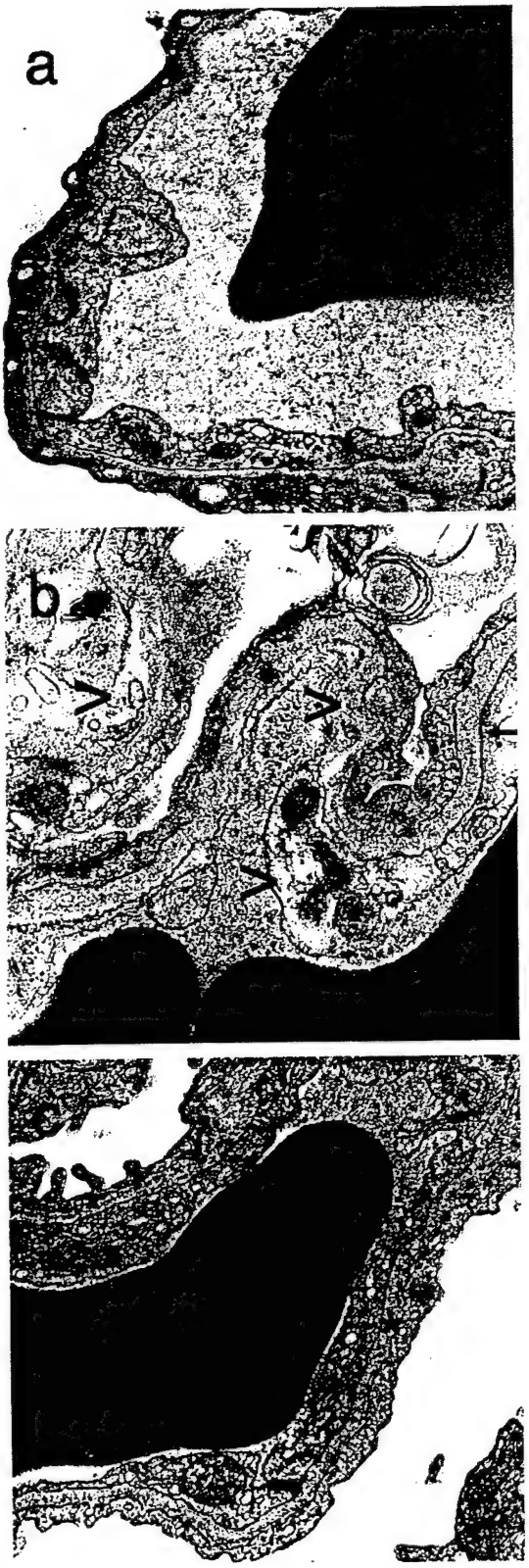


Figure 3. Nitrite + nitrate levels in the pleural effusion after IL-2 and L-NAME therapy in healthy mice ($10^6 \times M = \mu M$). Data represent mean ± SE (n = 3–5, each done in duplicate). * indicates significant difference from IL-2 treatment ($p < 0.05$). IL-2 (15,000 U/inj or 35,000 U/inj, i.p., every 8 h, 10 inj. total) induced dose dependent increases in nitrite + nitrate levels in the pleural effusion were significantly ($p < 0.05$) reduced with addition of L-NAME (0.1 mg/ml or more). L-NAME did not have any effects on the nitrite + nitrate levels induced by low IL-2 dose (7,500 U/inj). *Reproduced with kind permission from Orucevic and Lala J Immunother., 18: 210–220, 1996 Lipincott Raven Publishers.*

Increased levels of the final metabolites of NO (nitrates and nitrites) [82, 98] have been reported in human cancer patients receiving IL-2 therapy [30, 31, 99]. NO induction may be an indirect result of IL-2 therapy due to an induction of IFNγ and TNFα [15, 37]. Endothelial injury mediated by both of the cytokines has been linked with NO production [28, 29]. NO can contribute to capillary leakage by direct or indirect mechanisms. First, NO has been shown to mediate cytotoxicity in endothelial cells [28, 29] and thus cause a loss of integrity of the capillary lining. Second, high NO levels can indirectly enhance the capillary leakage in the lungs. It causes systemic hypotension [100] which in turn can indirectly cause pulmonary hypertension and thus increased pulmonary capillary pressure leading to further fluid leakage in the lungs.

Capillary leak syndrome results from numerous simultaneous or sequential events induced by IL-2 therapy: a hypothesis

In view of the literature reviewed earlier, it is reasonable to suggest that an increase in capillary permeability is caused by multiple factors initiated by high doses of IL-2. These factors operate by causing damage to capillary endothelial cells and/or by increasing capillary pressure. Capillary leak syndrome may result from numerous simultaneous or sequential events: 1) IL-2 induces LAK cell activation *in vivo* and promotes their adhesion to and subsequent cytotoxicity to endothelial cells; 2) IL-2 induces high levels of IFNγ which can change the cytoarchitecture of endothelial cells, making the endothelial lining more prone to leakage; 3) high levels of TNFα produced by IL-2-activated leuko-



cytes induces endothelial cell activation and adhesiveness for leukocytes and may play a role in increased capillary permeability; 4) NO induction at high levels remains at the end of the cascade of events induced by IL-2 therapy (e.g. production of TNFα and IFNγ) and plays a major role in capillary leakage both directly and indirectly, as discussed above.

Evidence for the central role of NO in the development of IL-2 therapy-induced capillary leakage in mice and its mitigation with NOS inhibitors

We conducted a series of studies to examine the role of NO in the pathogenesis of capillary leakage resulting from systemic IL-2 therapy in healthy and mammary adenocarcinoma-bearing C3H/HeJ mice. We measured IL-2 therapy-induced capillary leakage (pleural effusion, pulmonary edema and water retention in the spleen and the kidneys), NO production *in vivo* and the influence of treatment with NOS inhibitors (NMMA and N^G-Nitro L-Arginine methyl ester – L-NAME) on these parameters. Influence of these two inhibitors on IL-2 therapy-induced regression of the primary tumors and their lung metastases was also examined. In addition, the effects of these NOS inhibitors alone on

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Figure 4. Ultrastructure of the lungs of mice given IL-2 or IL-2 + L-NAME therapy. a = control b = IL-2; c = IL-2 + L-NAME; magnification × 20.000. Therapies were given in the following manner: IL-2 was given in a dose of 15,000 U/inj.i.p., every 8 h, 10 injections total: L-NAME was given in drinking water starting 1 d before IL-2 therapy.

Basement membrane is thick (→) and discontinuous in IL-2 treated mice. Endothelial as well as pneumocyte type I are severely damaged. There is also swelling of endothelial cells as well as pneumocyte type I. > indicates an area of blood-air barrier showing such damage. Addition of L-NAME therapy restored the ultrastructural integrity of alveoli and endothelium. Basement membrane is continuous and thin at the thin part of the capillary (*) in IL-2 + L-NAME treated animals. Endothelial cells, although in some cases remain swollen, are never detached from their basement membrane in these mice. *Reproduced with kind permission from Orucevic et al., Lab. Investigation, 76(1): 53–65, 1997. The United States and Canadian Academy of Pathology Inc.*

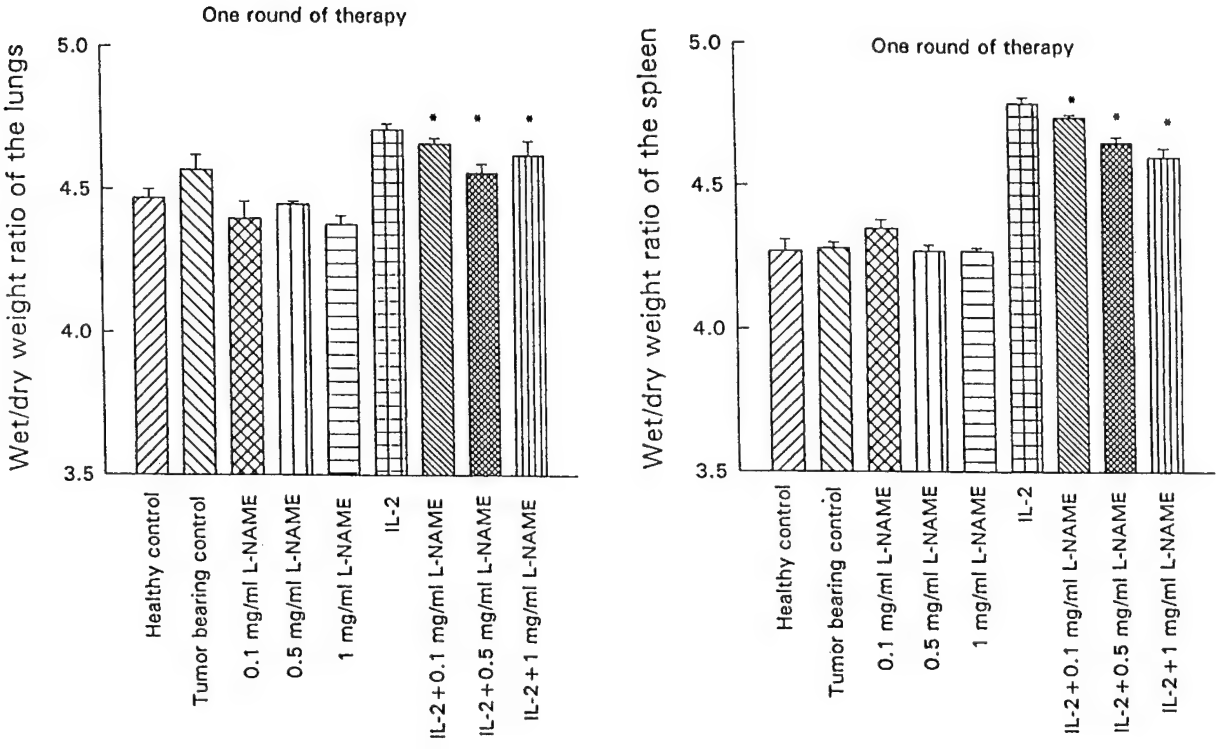

Figure 5. Water content of the lungs and spleen after IL-2 and L-NAME therapy in tumor-bearing mice. (left: lungs, right: spleen). Data represent mean ± SE (n = 10). Therapies were given in the following manner: IL-2 was given i.p. in a dose of 15,000 U/inj. every 8 h, 10 injections total, started 10 d after sc. inj of 250,000 C3–L5 mammary adenocarcinoma cells: L-NAME was given in drinking water starting on d 9 after tumor inoculation. * Addition of L-NAME significantly ($p < 0.05$) reduced IL-2 induced pulmonary edema after the first round of therapy.

* Addition of L-NAME significantly ($p < 0.001$) decreased IL-2 induced water retention in the spleen in a dose dependent manner after the first round of therapy. *Reproduced with kind permission from Orucevic and Lala. Br. J Cancer, 73: 189–196, 1996. Stockton Press, Hampshire, UK.*

mammary tumor growth and metastases were evaluated. Since L-NAME potentiated tumor-reductive effects of IL-2 therapy simultaneously with a reduction of IL-2-induced NO production *in vivo*, further experiments were designed to test whether L-NAME had a potentiating effect on IL-2 induced activation of antitumor effector cells *in vivo* and *in vitro*. This was tested by measuring antitumor cytotoxicity of splenocytes of healthy or tumor-bearing mice subjected to IL-2 ± L-NAME treatment *in vivo* and *in vitro*.

We initially tested whether treatment with NMMA can ameliorate IL-2 therapy-induced capillary leak syndrome in healthy or tumor-bearing mice without compromising the antitumor effects of IL-2 therapy [101]. We found that intraperitoneal IL-2 therapy caused substantial capillary leakage, both in healthy and tumor-bearing mice, as well as a substantial rise in NO production *in vivo* (measured in the serum and pleural effusion) in an IL-2 dose-dependent manner. Subcutaneously administered NMMA, when combined with IL-2 therapy, failed to ameliorate IL-2-induced capillary leakage in both groups of mice, and was also inadequate in significantly reducing IL-2 induced rise in NO production *in vivo*. It did not compromise anti-tumor effects of IL-2. In mammary adenocarcinoma bearing mice, subcutaneous NMMA therapy alone reduced tumor growth, spontaneous pulmonary metastasis and tumor-induced pulmonary edema.

This prompted us to test the effects of continuous oral administration of NMMA in healthy mice sub-

jected to IL-2 therapy. A substantial drop in NO production and capillary leakage was noted in these mice [101]. Since NO-blocking agents protected against IL-2-induced hypotension [80, 100], it was reasonable to expect that NMMA should also prevent IL-2 induced fluid leakage. It was evident that NMMA fulfilled this expectation only when given orally but not subcutaneously. This may be because the continuous oral administration of the drug was effective in blocking the rise in serum NO levels induced by IL-2 therapy, whereas the subcutaneous administration, in spite of repeated delivery, was inadequate in fully blocking NO production [101]. We suggested that the route of administration, as well as scheduling, were important determinants of therapeutic efficacy of NO inhibitors in the mitigation of IL-2 induced capillary leakage. This contention was supported by our findings that another NOS inhibitor, L-NAME also succeeded in mitigating IL-2 induced capillary leakage in healthy mice when given orally, but the benefits were only partial when given subcutaneously [102]. Similarly, attenuation of IL-2-associated capillary leakage was observed in another murine model by Samlowski *et al.* [103] when an NO inhibitor was given continuously in an osmotic minipump, whereas no effect on the IL-2-induced capillary leakage was noted by Leder *et al.* [104] when the NO inhibitor was given subcutaneously.

We did not test oral NMMA therapy in tumor-bearing mice, since L-NAME, another potent and less expensive NO inhibitor was soon available. Thus, we first tested whether L-NAME given chronically in the drinking water was effective in preventing capillary leakage induced by IL-2 therapy in healthy mice. We found that L-NAME was effective in preventing capillary leakage (pleural effusion – Figure 1, pulmonary edema – Figure 2 and water content of the spleen – not shown) induced by IL-2 therapy in healthy mice, and reduced IL-2-induced mortality when the IL-2 dose was not very high [102]. NO production appeared to be a strong determinant of the severity of this syndrome, because L-NAME treatment had a parallel effect in ameliorating the IL-2-induced capillary leakage and rise in NO production (Figure 3). A subsequent study [105] was designed in healthy C3H/HeJ mice

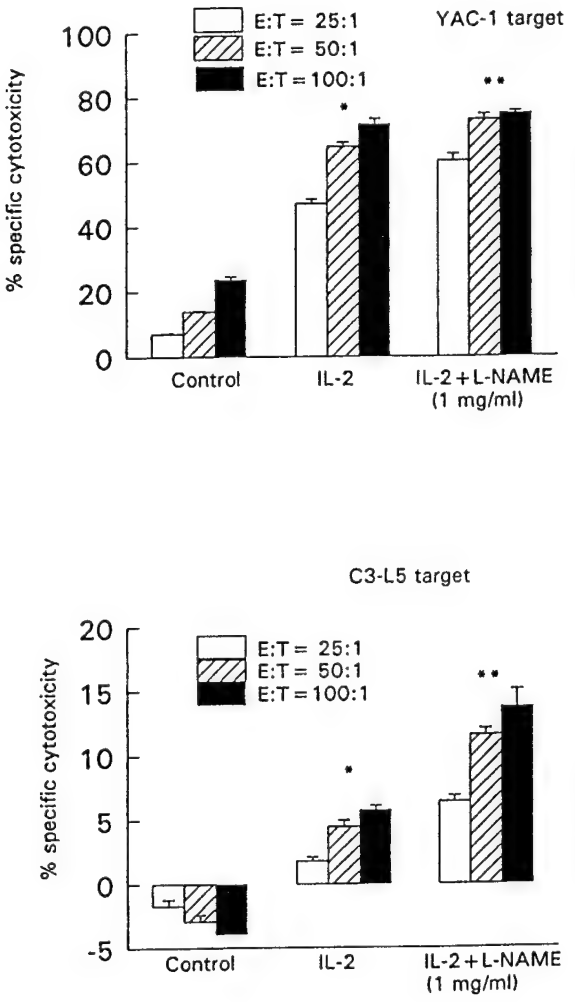

Figure 6. In vivo killer cell generation in healthy mice after IL-2 ± L-NAME therapy. Data represent mean ± SE (every effector: target ratio done in triplicate). * IL-2 therapy significantly ($p < 0.05$) improved splenocyte cytotoxicity (all three effector: target ratios combined) against NK sensitive-YAC-1 target and NK resistant – C3–L5 target. ** Addition of L-NAME therapy significantly ($p < 0.05$) enhanced IL-2 induced splenocyte cytotoxicity (all three effector: target ratios combined) against NK sensitive and NK resistant targets. *Reproduced with kind permission from Orucevic and Lala, Cellular Immunol., 169: 125–132, 1996, Academic Press, New York, USA.*

to (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) identify the histological nature of structural damage to the lungs during IL-2 therapy-induced pulmonary edema and (c) test whether the addition of L-NAME

therapy reduced the increase in NOS activity and IL-2-induced structural damage to the lungs. Morphological studies revealed that IL-2 therapy led to the induction of iNOS protein in numerous tissues, including the vascular endothelium, muscles of the anterior thoracic wall and splenic macrophages [105]. Biochemical studies revealed a positive association of high NOS activity in the lungs and the anterior thoracic wall with the presence of pulmonary edema, pleural effusion and structural damage to the lungs and its capillaries in IL-2 treated mice. Addition of L-NAME completely abolished the NOS activity, but not necessarily iNOS expression. It also reduced IL-2-induced pulmonary edema and pleural effusion, and significantly restored structural integrity of the lungs identified by light and electron microscopy (Figure 4) [105]. Thus, high tissue activity of IL-2-induced iNOS enzyme played a crucial role in the pathogenesis of IL-2-induced capillary leak syndrome.

Next, we tested whether L-NAME can prevent IL-2-induced capillary leakage in mammary adenocarcinoma bearing mice without compromising the therapeutic benefit of IL-2 [106]. In tumor-bearing mice, oral L-NAME therapy alone produced significant anti-tumor and anti-metastatic effects, similar to the effect noted earlier with NMMA therapy. L-NAME in combination with IL-2 therapy succeeded in ameliorating IL-2-induced as well as tumor-induced capillary leakage in tumor-bearing mice (Figure 5), and potentiated the tumor-reductive function of IL-2 [106]. Therefore, we tested whether a potentiation of IL-2-induced tumor regression by L-NAME therapy can be explained by a potentiation of LAK cell activation [107]. We found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2-induced generation of antitumor cytotoxicity of splenocytes of healthy (Figure 6) as well as mammary adenocarcinoma-bearing mice (not shown), concomitant with a drop in IL-2-induced NO production *in vivo* and *in vitro*. These results revealed that the IL-2-induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition with L-NAME therapy [107].

The above results provided the first direct evidence that NO is instrumental in IL-2-induced capillary leakage and that an NO blocking agent such as L-NAME can mitigate this leakage without interfering with the beneficial anti-tumor effects of IL-2 therapy. We also found that NO blocking agents alone can reduce tumor growth and spontaneous metastasis in this mammary tumor model in which tumor cells express eNOS. When combined with IL-2, NOS inhibitors improved IL-2 induced antitumor cytotoxicity, as well as tumor regression. Thus, NO blocking agents may be useful in treating tumors producing NO and serve as valuable adjuncts to IL-2 based therapies of cancer and infectious diseases.

Toxic side effects of systemic high dose IL-2 therapy, including capillary leakage, have recently forced investigators to seek alternate forms of IL-2 delivery, including gene therapy. These efforts have so far been less than promising in limited human trials. We propose that more research should be invested into combination therapies for achieving the dual benefit of amelioration of IL-2 toxicity and augmentation or the antitumor efficacy of systemic IL-2 therapy. A recent report [108] indicates that induction of oxygen-free-radicals may represent an additional arm of endothelial injury caused by IL-2 therapy, since treatment with dimethylthiourea (a scavenger of oxygen-free-radicals) attenuated IL-2 therapy-induced capillary leakage. We suggest that formation of peroxynitrite, a potent endotheliotoxic molecule, due to combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary damage. It remains to be seen whether combination therapies designed to block both NO and superoxide can provide better means of controlling IL-2 toxicity and improving antitumor effects of IL-2.

Key unanswered questions

1. Can more specific iNOS inhibitors such as L-N^6-(1-Iminoethyl)-lysine hydrochloride (NIL) [109] or 1400W [110] given continuously in an osmotic minipump provide better protection from IL-2 induced mortality in mice than L-NAME?
2. How does IL-2 therapy-induced NO mediate the damage to endothelial cells: by direct NO-mediated

apoptosis, or endothelial injury caused by an over production of peroxynitrite, or both?
3. Will the combination of an iNOS inhibitor and an oxygen – free – radical – scavenger (such as dimethylthiourea) prove to be superior to either of these agents alone in preventing IL-2 induced capillary leakage and mortality?

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NITRIC-OXIDE PRODUCTION BY MURINE MAMMARY ADENOCARCINOMA CELLS PROMOTES TUMOR-CELL INVASIVENESS

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The role of nitric oxide (NO) in tumor biology remains controversial and poorly understood. While a few reports indicate that the presence of NO in tumor cells or their micro-environment is detrimental for tumor-cell survival, and consequently their metastatic ability, a large body of data suggests that NO promotes tumor progression. The purpose of this study was to identify the source of NO in the spontaneously metastasizing C3-L5 murine mammary-adenocarcinoma model, the role of tumor-derived NO in tumor-cell invasiveness, and the mechanisms underlying the invasion-stimulating effects of tumor-derived NO. The source of NO was established by immunocytochemical localization of NO synthase (NOS) enzymes in C3-L5 cells *in vitro* and transplanted tumors *in vivo*. An *in vitro* transwell Matrigel invasion assay was used to test the invasiveness of C3-L5 cells in the presence or the absence of NO blocking agents or iNOS inducers (IFN-γ and LPS). The mechanisms underlying the invasion-stimulating effects of tumor-derived NO were examined by measuring mRNA expression of matrix metalloproteinases (MMP)-2 and -9, and tissue inhibitors of metalloproteinases (TIMP) 1, 2 and 3 in C3-L5 cells in various experimental conditions. Results showed that C3-L5 cells expressed high level of eNOS protein *in vitro*, and *in vivo*, both in primary and in metastatic tumors. C3-L5 cells also expressed iNOS mRNA and protein when cultured in the presence of IFN-γ and LPS. Constitutively produced NO promoted tumor-cell invasiveness *in vitro* by down-regulating TIMP 2 and TIMP 3. In addition, there was up-regulation of MMP-2, when extra NO was induced by IFN-γ and LPS. In conclusion, NO produced by C3-L5 cells promoted tumor-cell invasiveness by altering the balance between MMP-2 and its inhibitors TIMP-2 and 3. Thus, our earlier observations of anti-tumor and anti-metastatic effects of NO inhibitors *in vivo* in this tumor model can be explained, at least in part, by reduced tumor-cell invasiveness. *Int. J. Cancer* 81:889–896, 1999.

Nitric oxide (NO) is synthesized in mammalian cells from the amino acid L-arginine by a family of enzymes, the nitric-oxide synthases (NOS) (Knowles and Moncada, 1994; Billiar, 1995). This molecule plays a key role in many physiological as well as pathological processes, including inflammation and neoplasia. Numerous clinical and experimental studies indicate a contributory role to NO in tumor progression. The level of NOS protein and/or NOS activity has been positively correlated with the degree of malignancy in a number of human cancers, including human gynecological cancers (ovarian, uterine) (Thomsen *et al.,* 1994), central nervous system tumors (Cobbs *et al.,* 1995), breast cancer (Thomsen *et al.,* 1995), gastric cancer (Thomsen and Miles, 1998), squamous-cell carcinomas of the head and neck (Gallo *et al.,* 1998), prostatic cancer (Klotz *et al.,* 1998) and lung cancer (Fujimoto *et al.,* 1997). Aberrant NOS expression in the above cases has been explained by the presence of constitutive form(s) (eNOS, nNOS) in tumor cells and/or tumor endothelial cells, or the expression of the inducible form (iNOS) in tumor endothelial cells and/or tumor associated macrophages. Expression of iNOS in the tumor neovasculature has also been reported in experimental tumors (Buttery *et al.,* 1993; Kennovin *et al.,* 1994). Moreover, several lines of direct evidence exist for a facilitatory role of NO in tumor progression: (a) in a rat colonic-adenocarcinoma model, treatment with N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, reduced NO production as well as tumor growth (Kennovin *et al.,* 1994); (b) similarly, anti-tumor and anti-metastatic effects of 2 NOS inhibitors, N^G-methyl-L-arginine (NMMA) and L-NAME, have been observed in our laboratory using a mouse mammary-adenocarcinoma model (Orucevic and Lala, 1996*a,b;* Lala and Orucevic, 1998); (c) Edwards *et al.* (1996) observed that NO induced by lipopolysaccharide (LPS) and interferon gamma (IFN-γ) in EMT-6 murine mammary-carcinoma cells stimulated tumor growth and metastasis *in vivo,* despite NO-induced inhibition of cell growth *in vitro;* (d) numerous human colon-cancer cell lines were found to express NOS activity (Jenkins *et al.,* 1994), and engineered over-expression of iNOS in a human colonic-adenocarcinoma line increased tumor growth and vascularity when transplanted into nude mice (Jenkins *et al.,* 1995).

In apparent contradiction to the above reports, the presence of NAD(P)H diaphorase, NOS enzymes and NOS activity in human colonic mucosa, polyps and carcinomas appeared to be inversely related to colonic-tumor progression (Chhatwal *et al.,* 1994; Moochhala *et al.,* 1996). However, a subsequent study revealed high iNOS expression in human colonic adenomas, consistent with the notion that this facilitated their progression into carcinomas by stimulating angiogenesis (Ambs *et al.,* 1998). High NOS activity was inversely correlated to tumor growth and metastasis in a murine melanoma model (Xie *et al.,* 1995). Engineered over-expression of iNOS in the above melanoma cells (Xie *et al.,* 1995) or human renal-carcinoma cells (Juang *et al.,* 1998) was shown to reduce tumorigenicity, because of NO-mediated tumor-cell apoptosis. These reported discrepancies may be explained by the dual role of NO on tumor growth. Whereas very high NO-producing tumor-cell clones may delete themselves by apoptosis, lower levels of NO may facilitate *in vivo* growth of surviving clones by numerous mechanisms, including promotion of neo-angiogenesis, increased tumor blood flow, increased invasiveness, or by inhibition of apoptosis. While some evidence exists for a contributory role of NO in promotion of neo-angiogenesis (Buttery *et al.,* 1993; Ziche *et al.,* 1994, 1997*a,b;* Lala and Orucevic, 1998; Gallo *et al.,* 1998) and tumor blood flow (Andrade *et al.,* 1992), the possible role of tumor-derived NO on invasiveness has not been explored.

The mechanisms by which NO promotes growth or metastasis in our murine mammary-adenocarcinoma model (Orucevic and Lala, 1996*a,b*) remained poorly defined. The objectives of the present study were to identify the source of NO in this spontaneously metastasizing C3-L5 mammary-adenocarcinoma model, its role on tumor-cell invasiveness and the mechanisms underlying the observed invasion-stimulating effects of NO.

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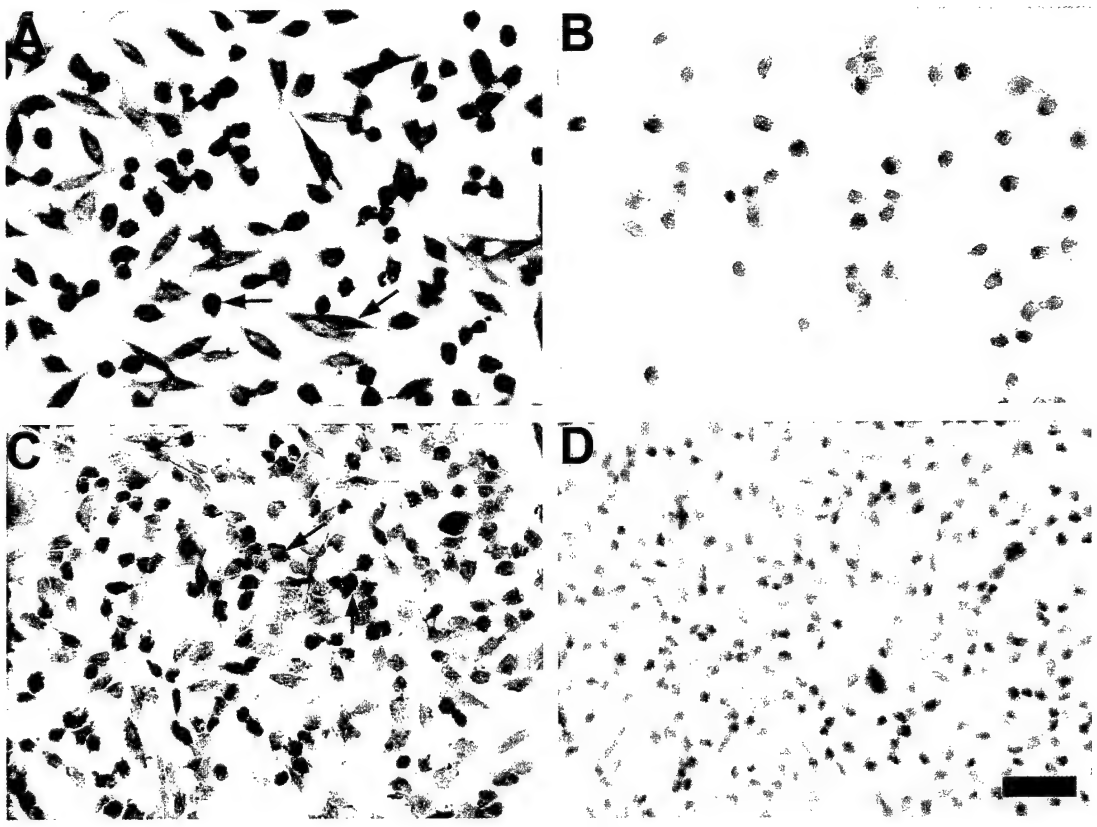


FIGURE 1 – Immunoperoxidase staining of C3-L5 cells in culture for NOS enzymes. *(a)* eNOS labelling is present in 90–95% of cells; *(b)* corresponding negative control (omission of primary antibody); *(c)* iNOS labelling is present in 20–25% of cells after induction with IFN-γ and LPS. Unstimulated cells showed no labelling for iNOS (data not shown); *(d)* corresponding negative control (omission of primary antibody). *(c)* and *(d)* were counterstained with hematoxylin. Scale bar: 50 μm.

MATERIAL AND METHODS

Tumor cell line and culture conditions

The C3-L5 mammary-adenocarcinoma cell line was selected and maintained in our laboratory from its parent C3 line, by 5 cycles of *in vivo* selection for spontaneous lung micrometastases following s.c. transplantation in C3H/HeJ mice (Lala and Parhar, 1993). C3 was a metastastic clone derived from a primary transplantable tumor T58, which had been isolated from a spontaneous mammary tumor in a C3H/HeJ retired female breeder mouse (Brodt *et al.*, 1985). Since the strong ability to spontaneously metastasize to the lungs, as originally exhibited by C3 cells, declined over the years of *in vitro* passages, re-selection was carried out *in vivo* to generate the C3-L5 line, which has since retained its ability to metastasize spontaneously to the lungs. C3-L5 cells were maintained in RPMI-1640 medium (GIBCO BRL, Burlington, Canada) with 1% penicillin/streptomycin (Mediatech, Washington, DC), supplemented with 10% FCS (GIBCO BRL). All experiments were done with cells passaged 3 to 5 times after thawing.

In experiments designed to block NO synthesis, L-NAME and NMMA (Sigma, St. Louis, MO) were added to the medium at various concentrations (0.01–1 mM); in some experiments, L-NAME was used at a concentration of 1 mg/ml of medium (equivalent to 3 mM). At all concentrations, there was no change in cell viability as measured by Trypan-blue exclusion.

In experiments designed to induce iNOS in tumor cells, a combination of recombinant murine IFN-γ (500–1000 U/ml) and LPS (10 μg/ml) was added to the culture medium. IFN-γ (lot FC2B11) was obtained from GIBCO, reconstituted with sterile water in aliquots of 10,000 U/100 μl and stored at −70°C until used for assays. LPS powder was obtained from Sigma, stored at 4°C, dissolved with complete medium on the day of the assay, and sterilized by filtration [0.2 μm filter pore size, Nalgene (Rochester, NY) syringe filters].

Immunocytochemical localization of NOS enzymes in C3-L5 cells in vitro

C3-L5 cells were grown for 24 hr on chamber slides, either in complete medium alone or in medium containing 500 U/ml of IFN-γ or 10 μg/ml of LPS or combination of IFN-γ and LPS in a humidified incubator (37°C, 5% CO_2 atmosphere). Slides were briefly washed with PBS, fixed in 10% buffered formalin and permeabilized with 0.25% Triton X-100 in PBS. After washing (3 × 5 min PBS), 10% normal goat serum was added to the slides as blocking serum for 1 hr. Slides were then subjected to the following treatments (30 min each) followed by washes: mouse monoclonal antibody against macrophage iNOS or endothelial NOS (eNOS) (Transduction Laboratories, Lexington, KY; 1:50 dilution), and secondary goat anti-mouse Ig biotinylated antibody (Dimension Laboratories, Mississauga, Canada; 1:200 dilution). Following treatment with ABC complex (1 hr) and DAB chromogen, slides were lightly counterstained with hematoxylin, and NOS expression was identified by positive brown staining. Negative controls were provided by omission of primary antibodies, or substitution of the primary antibodies by equivalent concentration of normal mouse Ig.

Immunohistochemical localization of NOS enzymes in C3-L5 cells in vivo

Samples of 5 primary tumors grown in C3H/HeJ mice for 24 days, following s.c. transplantation of 2.5×10^4 C3-L5 cells in the mammary line near the axilla, and of their spontaneous lung metastases were fixed in 10% buffered formalin, paraffin-embedded and cut into 5-μm-thick sections. After de-paraffiniza-

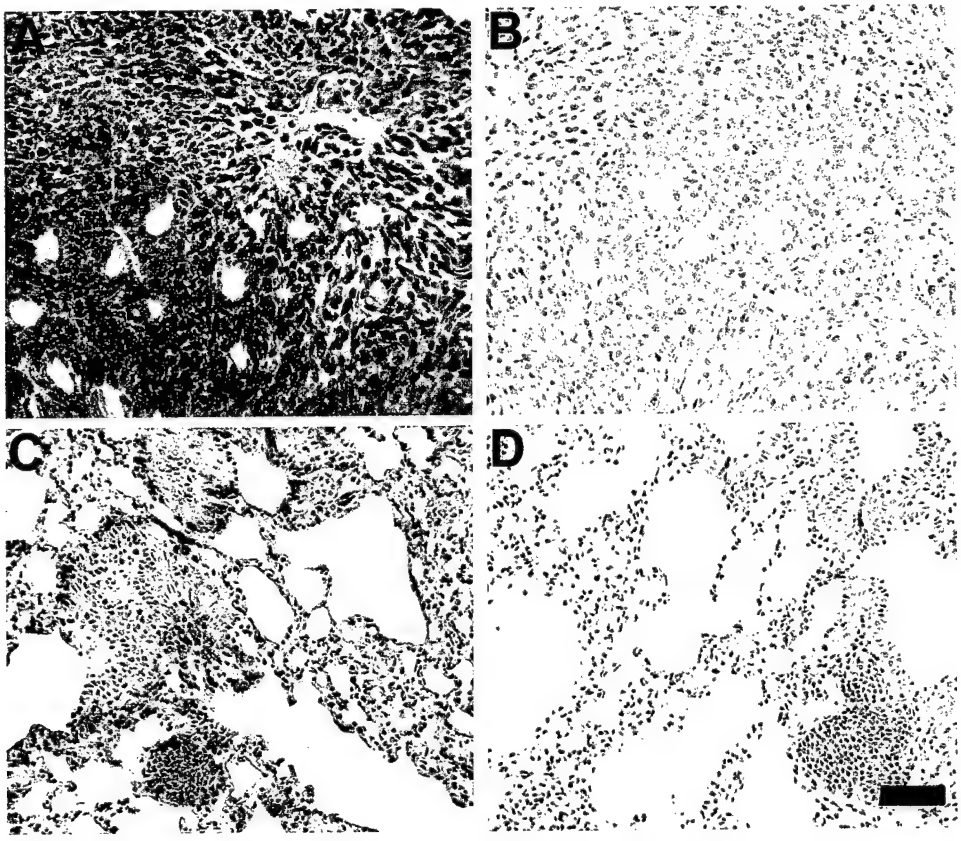


FIGURE 2 – Immunocytochemical localization of eNOS in primary tumors and their metastatic lung nodules, 24 days after s.c. transplantation of C3-L5 cells into C3H/HeJ mice. eNOS is present in approximately 80% of tumor cells at the primary site *(a)* and approximately 40–50% of tumor cells in a lung metastasis *(c)*. *(b)* and *(d)* are corresponding negative controls (omission of primary antibody). Sections were counterstained with hematoxylin. Scale bar: 50 μm.

tion and blocking of endogenous peroxidase activity (3% H_2O_2 in absolute methanol for 15 min), sections were permeabilized with 0.25% Triton X-100 in PBS. Normal horse serum (10%) or normal goat serum (10%) was added to the slides as blocking serum (1 hr), followed by mouse monoclonal primary antibody against iNOS or rabbit polyclonal antibody against eNOS (Affinity Bioreagents, Neshanic Station, NJ; 1:200 dilution) and incubated overnight at 4°C. Slides were then treated with secondary horse anti-mouse biotinylated antibody (rat absorbed IgG; Vector, Burlingame, CA; 1 in 200 dilution in 0.2% BSA in PBS) or goat anti-rabbit biotinylated antibody (as in earlier section) for 1 hr at room temperature, followed by ABC complex and DAB chromogen. Sections were then lightly counterstained with hematoxylin. Negative controls were provided by omission of primary antibodies, or substitution with normal mouse or rabbit Igs at equivalent concentrations.

Matrigel invasion by C3-L5 cells

An *in vitro* transwell Matrigel invasion assay devised in our laboratory (Graham *et al.*, 1993) was used to test the invasiveness of C3-L5 cells in the presence or absence of 0.01, 0.1, 1 and 3 mM NO blocking agent L-NAME or NMMA or iNOS inducers, IFN-γ (500–1000 U/ml) and LPS (10 μg/ml), in the presence or absence of L-NAME. Some wells contained excess L-arginine (5 times the concentration of NOS inhibitors, used as specificity control to abrogate the effects of NO inhibitors). In this assay, tumor cells were pre-labelled with ^{3}HTdR for 24 hr, then added to the invasion chamber of the transwell containing a millipore membrane coated with Matrigel (reconstituted basement membrane; Collaborative Research, Bedford, MA). Percentages of labelled cells penetrating the Matrigel-millipore membrane were scored as percent radioactivity appearing in the lower well and bottom of the millipore membrane, as a function of time (1–3 days). All assays were done in triplicate.

Measurement of NO production in the media from C3-L5 cells

C3-L5 cells were grown in 24-well plates (10^6 cells/800 μl media/well) in triplicate, in the medium alone, in L-NAME alone (1 mg/ml) or in the presence of IFN-γ (500–1000 U/ml) + LPS (10 μg/ml) ± L-NAME. Culture media from wells were collected after 24 hr of the incubation period, and kept frozen at −20°C, until assayed for NO_2^- levels. Griess reagent (Green *et al.*, 1982) was used for measurement of NO_2^-.

Analysis of mRNA expression for matrix metalloproteases 2 and 9 (MMP-2, MMP-9) and tissue inhibitors of metalloproteases (TIMP) 1, 2 and 3 in C3-L5 cells

C3-L5 cells were grown for 24 hr on 10-cm^2 tissue-culture dishes, either in RPMI medium with 1% BSA or in the medium with NMMA or IFN-γ + LPS ± NMMA. Total RNA was isolated by standard methods (Chomczynski and Sacchi, 1987) using RNAzol B (Biotecx, Houston, TX). Total RNA (20 μg) from each sample was electrophoresed on a 1% agarose gel containing 3% formaldehyde prior to transfer to Gene Screen membrane (DuPont-NEN, Boston, MA) and UV autocrosslinked (UV Stratalinker 1800, Stratagene, La Jolla, CA). cDNA probes for TIMP 1, 2 and 3 were obtained from Dr. R. Khokha (Ontario Cancer Institute, Toronto, Canada) and murine MMP-2 (72-kDa collagenase) and MMP-9 (92-kDa collagenase) probes were obtained from Dr. D. Edwards (University of Calgary, Canada). cDNA probe for murine

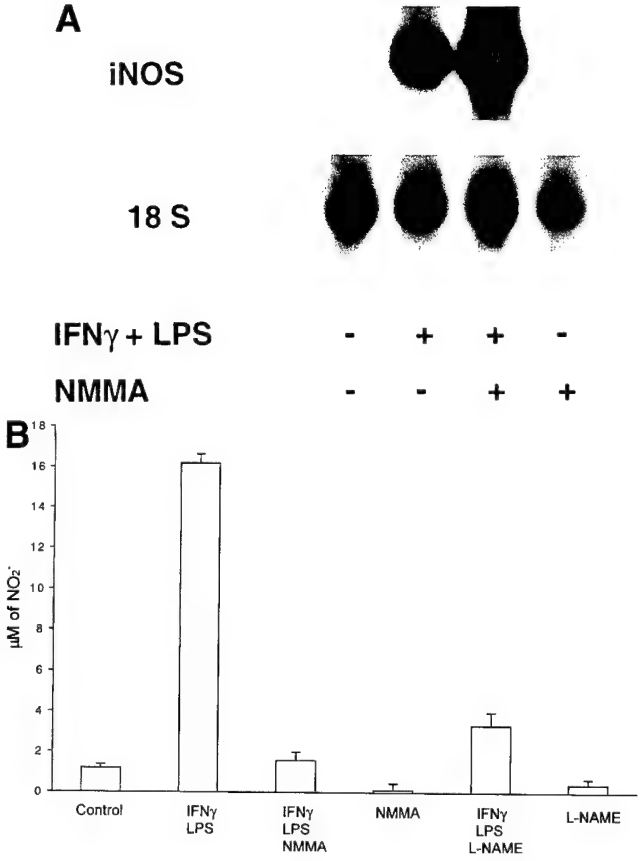


FIGURE 3 – iNOS induction and NO production following treatment of C3-L5 cells with IFN-γ and LPS. *(a)* mRNA expression of iNOS. iNOS was not expressed by C3-L5 cells in native condition, but was induced by IFN-γ and LPS. This induction was up-regulated by treatment of cells with NMMA. *(b)* NO_2^- levels in the medium of C3-L5 cells after 24-hr treatment with IFN-γ and LPS ± NMMA or L-NAME. Data represent mean (of triplicate determinations) ± S.E. IFN-γ and LPS treatment induced significant increase in NO production. Addition of NMMA or L-NAME significantly reduced NO_2^- levels in the medium, but not to the control levels.

iNOS was obtained from Dr. C. Lowenstain (Johns Hopkins University, Baltimore, MD). These cDNA probes were labeled with [^{32}P]dCTP by random priming. Hybridization was carried out overnight at 43°C and hybridized filters were washed at 53°C, following methods from published protocols (Geller *et al.,* 1995). Autoradiography was performed at −70°C in the presence of intensifying screen. Northern-blot membranes were stripped for re-hybridization with 18s rRNA utilized as loading controls. Relative mRNA levels were quantitated by PhosphorImager scanning using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

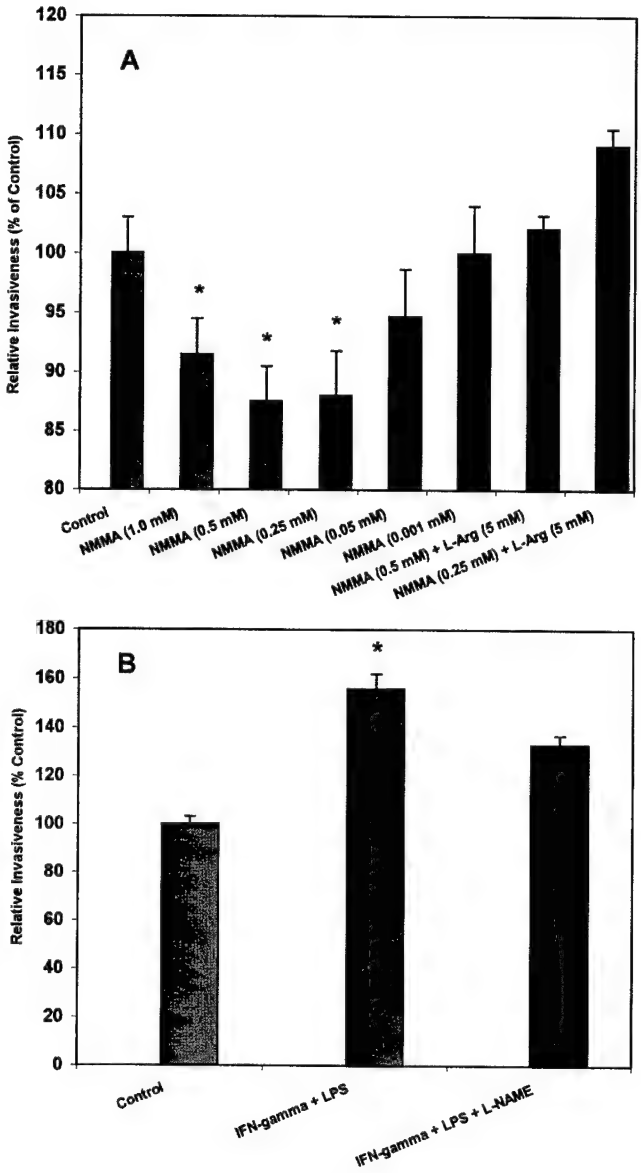
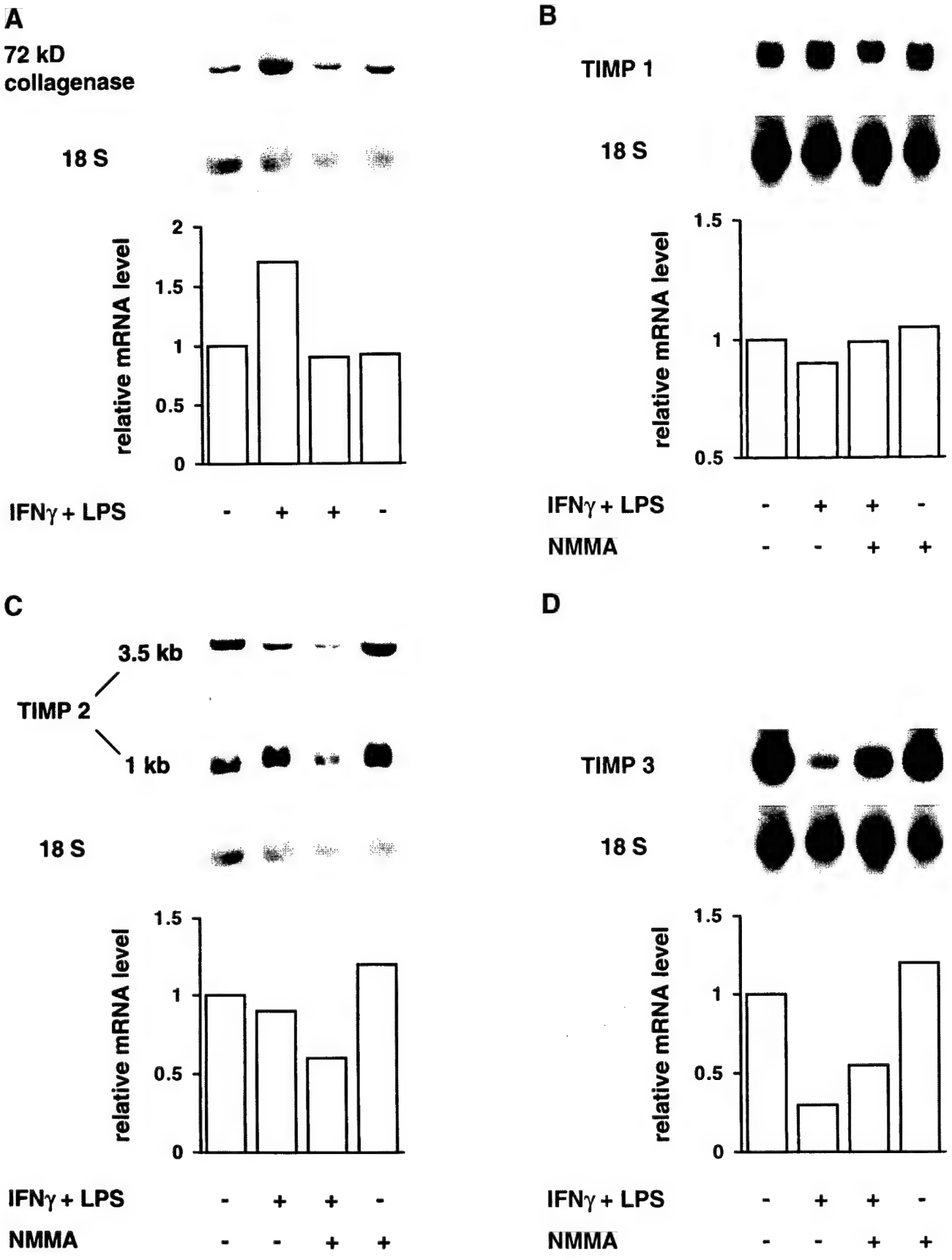


FIGURE 4 – Matrigel invasion by C3-L5 cells treated with different concentrations of NMMA (± L-arginine) *(a)* or IFN-γ and LPS (± L-NAME) *(b)*. Data represent mean of triplicate determinations ± S.E. NMMA at concentrations of 0.25–1 mM reduced tumor-cell invasiveness ($p < 0.05$). L-arginine (5-fold excess) abrogated the NMMA effects, indicating the specificity of NMMA action. IFN-γ (500 U/ml) and LPS (1 mg/ml) significantly stimulated invasiveness of C3-L5 cells ($p < 0.05$). This stimulation was marginally abrogated ($p = 0.055$) by addition of L-NAME. *Significantly different ($p < 0.05$) from control.

FIGURE 5 – Northern blots of mRNA expression of MMP-2 and TIMP-1, 2, and 3 by C3-L5 cells in different conditions. Data indicate representative results from 1 of 2 separate experiments. MMP-9 (92-kDa collagenase) mRNA expression was absent in these cells (data not shown). *(a)* mRNA expression of MMP-2 (72-kDa collagenase). IFN-γ and LPS treatment up-regulated MMP-2 mRNA expression in C3-L5 cells, and addition of NMMA to this treatment restrained collagenase-mRNA expression to the control level. NMMA alone did not have any effect on MMP-2 expression. *(b)* mRNA expression of TIMP-1. IFN-γ and LPS treatment with or without NMMA, or NMMA treatment alone, did not significantly influence TIMP-1 mRNA expression. *(c)* mRNA expression of TIMP-2. IFN-γ and LPS treatment down-regulated TIMP-2 mRNA expression. Addition of NMMA to LPS and IFN-γ did not restore TIMP-2 expression. NMMA treatment alone, however, up-regulated expression of TIMP-2. *(d)* mRNA expression of TIMP-3. IFN-γ and LPS treatment down-regulated TIMP-3 mRNA expression. Addition of NMMA to LPS and IFN-γ partially restored TIMP-3 expression. NMMA treatment alone, however, up-regulated expression of TIMP-3.


A
72 kD collagenase
18 S
relative mRNA level
2
1.5
1
0.5
0
IFNγ + LPS - + + -
B
TIMP 1
18 S
relative mRNA level
1.5
1
0.5
IFNγ + LPS - + + -
NMMA - - + +
C
3.5 kb
TIMP 2
1 kb
18 S
relative mRNA level
1.5
1
0.5
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IFNγ + LPS - + + -
NMMA - - + +
D
TIMP 3
18 S
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1.5
1
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FIGURE 5.

Statistics

Data were analyzed using one-way analysis of variance. Differences were considered significant at $p \leq 0.05$.

RESULTS

Expression of immunoreactive eNOS protein by C3-L5 cells in vitro *and* in vivo

Immunocytochemical staining for eNOS enzyme revealed that C3-L5 mammary-carcinoma cells constitutively expressed high levels of eNOS protein *in vitro* (Fig. 1*a*).

S.c. C3-L5 tumors grown in C3H/HeJ mice for 24 days, also their spontaneous metastatic counterparts (lungs) expressed eNOS protein (Fig. 2*a,c*). eNOS protein was present, on average, in approximately 80% of tumor cells at the primary tumor site and in about 40 to 50% of the tumor cells in the lung metastatic nodules harvested at 24 days, indicating that eNOS expression may be somewhat down-regulated during tumor growth at the metastatic site.

Expression of iNOS in C3-L5 cells in vitro

C3-L5 cells did not express iNOS mRNA under native conditions; however, iNOS mRNA was induced by 24-hr stimulation with IFN-γ and LPS (500 U/ml and 10 μg/ml, respectively). This induction was up-regulated by treatment of cells with NMMA (Fig. 3*a*). IFN-γ and LPS treatment induced significant increase in NO production, measured as NO_2^- levels in the medium. Additional presence of NMMA or L-NAME significantly reduced NO_2^- levels in the medium, but not to the control levels (Fig. 3*b*). Expression of immunoreactive iNOS protein after induction with the combination of IFN-γ and LPS (Fig. 1*c*) was correlated with mRNA expression. IFN-γ alone or LPS alone did not induce iNOS mRNA or protein in C3-L5 cells (data not presented).

Invasiveness of C3-L5 cells in vitro

C3-L5 cells, on their own, exhibited significant invasiveness in Matrigel invasion assay, as indicated by invasion indices ranging between 30 and 50% in different experiments. These values were normalized to 100% in Figure 4, to indicate the effects of NOS inhibitors (NMMA, L-NAME) or iNOS inducer (IFN-γ and LPS). NMMA at 0.25- to 1-mM concentrations reduced the invasiveness of C3-L5 cells. Addition of excess L-arginine abrogated the NMMA effects, indicating the specificity of NMMA action (Fig. 4*a*). Another NOS inhibitor, L-NAME, at concentrations of 0.01 to 1 mM also significantly reduced invasiveness of C3-L5 cells ($p < 0.05$) in a Matrigel invasion assay (data not shown).

Combination of IFN-γ and LPS (500 U/ml and 10 μg/ml respectively) significantly stimulated invasiveness of C3-L5 cells ($p < 0.05$) in a 3-day Matrigel invasion assay (Fig. 4*b*). Although the presence of L-NAME (1 mg/ml or 3 mM) did not abrogate this stimulation significantly, a trend was noted (Fig. 4*b*; $p = 0.055$). A combination of IFN-γ and LPS at higher concentrations (1000 U/ml and 10 μg/ml respectively), also significantly stimulated invasiveness of C3-L5 cells in 1-day and 3-day Matrigel invasion assays in separate experiments, in spite of the fact that at these concentrations there was a small drop in cell viability at day 3, most likely due to NO-mediated apoptosis (data not presented).

mRNA expression of MMPs and TIMPs in C3-L5 cells treated with IFN-γ and LPS, and with NMMA

IFN-γ and LPS treatment up-regulated MMP-2 (72-kDa collagenase) mRNA expression in C3-L5 cells, and addition of NMMA to this treatment restrained mRNA expression to the control level. NMMA alone did not have any effect on MMP-2 mRNA expression (Fig. 5*a*). C3-L5 cells did not express MMP-9 (92-kDa collagenase) mRNA in any conditions (data not shown).

IFN-γ and LPS treatment with or without NMMA, or NMMA treatment alone, did not significantly influence TIMP 1 mRNA expression (Fig. 5*b*).

IFN-γ and LPS treatment caused minor down-regulation of TIMP-2 mRNA (Fig. 5*c*), and strong down-regulation of TIMP-3 mRNA (Fig. 5*d*) expression. Addition of NMMA to LPS and IFN-γ partially restored TIMP-3 expression (Fig. 5*d*), but TIMP-2 mRNA expression remained suppressed. NMMA treatment alone resulted in slight up-regulation of TIMP-2 and TIMP-3 mRNA expression (Fig. 5*c,d*).

DISCUSSION

Results from the present study revealed that *in vitro*-propagated C3-L5 mammary-adenocarcinoma cells expressed eNOS protein and, in addition, were stimulated to express iNOS protein, when grown in the presence of IFN-γ and LPS. Tumor cells grown *in vivo* expressed eNOS, but not iNOS protein, both at the primary site, as well as the sites of spontaneous lung metastasis. These cells exhibited a strong ability to invade Matrigel, and their invasiveness was reduced in the presence of NO-blocking agents (NMMA and L-NAME). This was paralleled by up-regulation of TIMP-2 and TIMP-3 mRNA expression. The anti-invasive effects of NOS inhibitors were noted in the absence of any change in cell viability (data not presented), and were abrogated in the presence of excess L-arginine, attesting to fact that the effects were due to inhibition of NO synthesis. Finally, invasiveness of C3-L5 cells was stimulated in the presence of iNOS-inducing agents IFN-γ and LPS, with concurrent expression of iNOS protein and increase in NO production *in vitro*. This was paralleled by up-regulation of MMP-2 mRNA and down-regulation of TIMP-2 and TIMP-3 mRNA. In this case, addition of NOS inhibitor failed to abrogate the invasiveness significantly but did, however, restore 72-kDa collagenase expression to the control level and only partially restored TIMP-3 but not TIMP-2 expression. Taken together, these results demonstrated that NO production by C3-L5 cells promoted tumor-cell invasiveness by altering the balance between expression of MMP-2 and its inhibitors TIMP 2 and TIMP 3. Further work is needed to demonstrate a corresponding shift in the activities of the enzyme and its inhibitors at the protein level.

We have reported (Orucevic and Lala, 1996,*a,b*) that treatment of C3H/HeJ mice bearing C3-L5 mammary-adenocarcinoma transplants with NOS inhibitors (NMMA and L-NAME) had significant anti-tumor and anti-metastatic effects. Growth-retarding effects of L-NAME were also observed in a rat colonic-adenocarcinoma model (Kennovin *et al.,* 1994), indicating that NO promoted tumor progression in these tumor models. Indeed, high NOS activity or NOS protein expression has been positively correlated to the progression of tumors of the human reproductive tract (Thomsen *et al.,* 1994), mammary gland (Thomsen *et al.,* 1995), stomach (Thomsen and Miles, 1998), central nervous system (Cobbs *et al.,* 1995), pharynx (Gallo *et al.,* 1998), prostate (Klotz *et al.,* 1998) and the lungs (Fujimoto *et al.,* 1997). In concurrence with these observations, the highly metastatic C3-L5 mammary-carcinoma cell line used in the present study was found to express high levels of eNOS protein *in vitro,* as well as *in vivo* in primary and metastatic tumors. C3-L5 cells also expressed iNOS protein following culture with IFN-γ and LPS. These findings attest to the NO-producing ability of these cells in constitutive conditions, which may be enhanced in inductive circumstances.

Multiple mechanisms may be postulated for the role of NO produced by tumor cells or host-derived cells in promoting tumor growth or metastases. Because of its vasodilatory function (Palmer *et al.,* 1987), NO may promote blood flow through the tumor vasculature and thus indirectly promote tumor-cell nourishment. This hypothesis has been validated in a rat adenocarcinoma model (Kennovin *et al.,* 1994), as well as in numerous other experimental tumors (Fukumura and Jain, 1998). NO has been shown to have a stimulatory effect on angiogenesis *in vitro* (Ziche *et al.,* 1994), as well as *in vivo,* when tested with a rabbit cornea model (Ziche *et al.,* 1994, 1997*a*), or a model of healing gastric ulcer (Konturek *et al.,*

1993). The angiogenesis-promoting role of NO has also been substantiated in a number of tumor models: (a) increased vascularity of transplants of human colonic-adenocarcinoma cells in nude mice, when these cells were engineered to over-express mouse iNOS gene (Jenkins *et al.,* 1995); (b) abrogation of C3-L5 murine mammary-tumor-induced angiogenesis in a Matrigel-implant assay, when the matrigel-implanted mice were subjected to L-NAME therapy (Lala and Orucevic, 1998; Jadeski and Lala, 1998); (c) reduction of angiogenesis in the rabbit cornea, induced by implantation of NO-producing human squamous-cell carcinomas when the rabbits were subjected to L-NAME therapy (Gallo *et al.,* 1998).

Another possible mechanism, direct NO-mediated stimulation of tumor-cell proliferation, has been excluded in our C3-L5 tumor model. Treatment of C3-L5 cells *in vitro* with NMMA had no effect on ^{3}HTdR uptake by these cells (data not shown). Finally, as demonstrated in this study, NO production by C3-L5 cells promoted tumor-cell invasiveness, and this mechanism may explain, at least in part, the observed reduction of primary tumor growth, as well as spontaneous lung metastasis following NMMA or L-NAME therapy (Orucevic and Lala, 1996*a,b*). Indeed, only modest anti-invasive effects of NOS inhibitors noted *in vitro* suggest that the *in vivo* anti-tumor action of the inhibitors in this tumor model must involve additional mechanisms such as reduced angiogenesis.

The mechanisms underlying the invasion-stimulating effects of NO in our tumor model appear to be, at least partly, due to an alteration in the balance between MMPs and their inhibitors. While endogenous, constitutive NO production by tumor cells caused down-regulation of TIMP-2 and TIMP-3, additional NO production under inductive conditions led to up-regulation of MMP-2. Such inductive conditions may occur *in vivo* during cytokine therapy of cancer and may counter some of the beneficial anti-tumor effects of cytokine therapy, such as tumor-cell death resulting from NO-mediated apoptosis or activation of anti-tumor effector cells.

Addition of NOS inhibitors to the NO inducer IFN-γ and LPS only partially abrogated C3-L5 tumor-cell invasiveness. There are several possible explanations: (a) incomplete inhibition of NO production, associated with sustained suppression of TIMP-2, in spite of restoration of MMP-2 expression to normal, as demonstrated in the results; (b) other NO-independent pathway(s) of invasion stimulation by IFN-γ and LPS.

Additional mechanisms underlying NO-mediated stimulation of cellular invasiveness may exist. For example, NO promotes degradation of articular cartilages by stimulating other MMPs (collagenases and stromelysin) in human, bovine or rabbit chondrocytes (Murrell *et al.,* 1995; Tamura *et al.,* 1996). In addition, it has been reported that TIMP-1 protein can be inactivated by peroxynitrite, which is formed rapidly from NO and O_2^- in conditions such as inflammation and ischemic re-perfusion (Frears *et al.,* 1996). Finally, NO has been shown to up-regulate urokinase-type-plasminogen activator (uPA) in endothelial cells of post-capillary venules during the process of NO-mediated stimulation of angiogenesis (Ziche *et al.,* 1997*b*). Since uPA converts plasminogen to plasmin, which can activate numerous MMPs, this may represent another pathway of NO-mediated stimulation of matrix degradation.

In apparent contrast to the above studies and the data presented here, engineered over-expression of iNOS in K1735 murine melanoma cells leading to decreased tumor-cell survival and tumorigenicity (Xie *et al.,* 1995) was reported to be associated with down-regulation of MMP-2 (Xie and Fidler, 1997) owing to down-regulation of its promoter activity. The reasons for this discrepancy remains unclear. Furthermore, additional biological roles of TIMP-1, TIMP-2 and TIMP-3 other than protease inhibition should not be disregarded. These molecules were reported to act as growth-promoting or -inhibiting proteins depending on the cell type (Bertaux *et al.,* 1991; Hayakawa *et al.,* 1992; Murphy *et al.,* 1993; Nemeth and Goolsby, 1993; Bian *et al.,* 1996; Sun *et al.,* 1996). Thus, further studies are needed to evaluate the biological consequences of NO-mediated regulation of MMPs and TIMPs in other tumor-cell systems. It is also possible that the genetic make-up of tumor cells may influence the biological role of NO in tumor progression (Ambs *et al.,* 1997; Lala and Orucevic, 1998). For example, expression of functional p53 in conjunction with iNOS was shown to promote NO-mediated apoptosis owing to *p53*-mediated accumulation of iNOS protein, whereas mutation or loss of *p53* provided a dual advantage to tumor cells: resistance to NO-mediated apoptosis, and increased tumorigenicity *in vivo* due to increased vascularity (Ambs *et al.,* 1997).

In conclusion, NO-mediated promotion of tumor-cell invasiveness resulting from an altered balance between MMP-2 and its inhibitors, in conjunction with the angiogenesis-stimulating role of NO, may account for the anti-tumor and anti-metastatic effects of therapy with NOS inhibitors in the C3-L5-mammary-adenocarcinoma model.

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Animal Model

Nitric Oxide Synthase Inhibition by N^G-Nitro-L-Arginine Methyl Ester Inhibits Tumor-Induced Angiogenesis in Mammary Tumors

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Using a murine breast cancer model, we earlier found a positive correlation between the expression of nitric oxide synthase (NOS) and tumor progression; treatment with inhibitors of NOS, N^G-methyl-L-arginine (NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME), had antitumor and antimetastatic effects that were partly attributed to reduced tumor cell invasiveness. In the present study, we used a novel *in vivo* model of tumor angiogenesis using subcutaneous implants of tumor cells suspended in growth factor-reduced Matrigel to examine the angiogenic role of NO in a highly metastatic murine mammary adenocarcinoma cell line. This cell line, C3L5, expresses endothelial (e) NOS *in vitro* and *in vivo*, and inducible (i) NOS *in vitro* on stimulation with lipopolysaccharide and interferon-γ. Female C3H/HeJ mice received subcutaneous implants of growth factor-reduced Matrigel inclusive of C3L5 cells on one side, and on the contralateral side, Matrigel alone; L-NAME and D-NAME (inactive enantiomer) were subsequently administered for 14 days using osmotic minipumps. Immediately after sacrifice, implants were removed and processed for immunolocalization of eNOS and iNOS proteins, and measurement of angiogenesis. Neovascularization was quantified in sections stained with Masson's trichrome or immunostained for the endothelial cell specific CD31 antigen. While most tumor cells and endothelial cells expressed immunoreactive eNOS protein, iNOS was localized in endothelial cells and some macrophages within the tumor-inclusive implants. Measurable angiogenesis occurred only in implants containing tumor cells. Irrespective of the method of quantification used, tumor-induced neovascularization was significantly reduced in L-NAME-treated mice relative to those treated with D-NAME. The quantity of stromal tissue was lower, but the quantity of necrotic tissue higher in L-NAME relative to D-NAME-treated animals. The total mass of viable tissue (ie, stroma and tumor cells) was lower in L-NAME relative to D-NAME-treated animals. These data suggest that NO is a key mediator of C3L5 tumor-induced angiogenesis, and that the antitumor effects of L-NAME are partly mediated by reduced tumor angiogenesis. *(Am J Pathol 1999, 155:1381–1390)*

Nitric oxide, an inorganic free radical gas, is synthesized from the amino acid L-arginine by a group of enzymes, the NO synthases (NOS). At least three isoforms of NOS have been cloned, characterized, and localized: endothelial (e) and neuronal (n) NOS isoforms are Ca^{2+}/calmodulin-dependent and are expressed constitutively in these and other cells. The inducible (i) isoform is Ca^{2+}/calmodulin-independent and usually induced in the presence of inflammatory cytokines and bacterial products in macrophages, hepatocytes, and other cells. Under certain conditions, iNOS can also be expressed constitutively in some cells. When constitutively expressed, NO produced at low levels is an important mediator of physiological functions such as vasodilation, inhibition of platelet aggregation, and neurotransmission. Under inductive conditions, high levels of NO produced by macrophages and other effector cells can mediate antibacterial and antitumor functions. However, chronic induction of NOS may contribute to many pathological processes including inflammation and cancer.[1,2]

Much scientific research has focused on the role of NO in tumor progression; although two apparently conflicting views exist, overall an overwhelming amount of clinical and experimental evidence supports a positive association between NO production and tumor progression. The level of NOS protein and/or activity in the tumor has been positively correlated with the degree of malignancy for

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tumors of the human reproductive tract,[3] breast,[4,5] and central nervous system.[6] In a majority of gastric carcinomas, iNOS was detected in stromal elements, and eNOS was detected in the tumor vasculature.[7] iNOS expression was higher in prostatic carcinomas relative to benign prostatic hyperplasia.[8] Similarly, relative to normal healthy control tissue, total NOS activity was higher in carcinomas of the larynx, oropharynx, oral cavity,[9] and adenocarcinomas of the lung.[10]

Experimental tumor models have provided more direct evidence of a contributory role of NO in tumor progression. Using a rat adenocarcinoma model in which cells of the tumor vasculature expressed iNOS, treatment of the host with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) reduced NO production and tumor growth.[11] Furthermore, despite *in vitro* cytostatic effects of NO induction with lipopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine mammary cells, this induction stimulated tumor growth and metastasis *in vivo*.[12] Finally, in our own studies using a murine mammary adenocarcinoma model (C3H/HeJ spontaneous mammary tumors and their clonal derivatives), NO-mediated stimulation of tumor progression was observed.[13] The spontaneously developing tumors showed heterogeneous expression of eNOS within primary tumors, whereas their metastatic counterparts were homogeneously eNOS positive, suggesting that eNOS expression promoted metastasis. A highly metastatic cell line, C3L5, clonally derived from a spontaneous mammary tumor showed strong eNOS expression *in vitro* and *in vivo*, and iNOS *in vitro* on stimulation with LPS and IFN-γ.[13] Treatment of C3L5 mammary tumor-bearing mice with inhibitors of NOS, L-NAME and N^G-methyl-L-arginine (NMMA), had antitumor and antimetastatic effects.[14,15] Reduced tumor cell invasiveness was identified as one of the mechanisms mediating these effects.[13,16] We hypothesized that, in this tumor model, additional mechanisms likely played critical roles in mediating the therapeutic effects of NOS inhibition.

In contrast to the above, some studies reported an inverse association between NO and tumor progression. For example, the levels of NOS enzymes and NOS activity declined during the transition of human colonic mucosa to polyps, and then to carcinomas.[17] However, a later study revealed higher NOS activity in adenomatous polyps, which was believed to promote increased angiogenesis before the transition of adenomas into carcinomas.[18] In a murine melanoma cell line, NOS activity was inversely correlated with capacity for metastasis.[19] When genetically transduced to overexpress iNOS, the melanoma cells,[19] as well as renal carcinoma cells,[20] lost their tumorigenic and metastatic abilities as a result of NO-mediated tumor cell apoptosis. These opposing findings suggest a dual role for NO in tumor growth and metastasis; the susceptibility of tumor cells to NO-mediated injury may depend on levels of NO produced and the genetic makeup of the tumor cells. During clonal evolution of tumors, high NO-producing cells may self-delete by apoptosis, and those making lower levels of NO or capable of resisting NO-mediated injury may have an *in vivo* advantage, resulting from NO-mediated stimulation of tumor cell invasiveness, tumor blood flow or tumor angiogenesis.[13]

A body of recent evidence suggests a stimulatory role of NO in angiogenesis. For example, NO donors were found to increase proliferation and migratory function of endothelial cells *in vitro*.[21,22] Using the *in vivo* rabbit cornea assay, angiogenesis induced by vasoactive molecules such as substance P and prostaglandin E_1 was blocked with NOS inhibition.[22] Similarly, NOS inhibitors reduced neovascularization in acetic acid-induced gastric ulcers in rats,[23] and human squamous cell carcinoma xenografts in the rabbit cornea.[9] An angiogenesis-promoting role for tumor-derived NO was also suggested by the *in vivo* behavior of a human colon adenocarcinoma cell line engineered to continuously express iNOS. When transplanted into nude mice, the iNOS-transduced cells resulted in tumors with enhanced growth rate and vascularity relative to those derived from wild-type control cells.[24]

In the present study, we have evaluated the contributory role of NO in C3L5 mammary tumor-induced angiogenesis. To achieve this, we devised a novel *in vivo* Matrigel implant model of tumor-induced angiogenesis and subjected the host animals to chronic treatment with the NOS inhibitor L-NAME, or as controls, its inactive enantiomer, D-NAME.

Materials and Methods

Animals

Female C3H/HeJ mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). On arrival at the vivarium, animals were immediately randomized to treatment groups (ie, L-NAME and D-NAME); experimental procedures began after a one-week acclimatization period. Throughout the investigation, animals had free access to food (standard mouse chow) and water and were maintained on a 12-hour light/dark cycle. Animals were treated in accordance with guidelines set out by the Canadian Council on Animal Care.

Tumor Cell Line

A spontaneously occurring mammary tumor in a female retired breeder C3H/HeJ mouse was the source of a primary transplantable tumor T58 from which a metastatic C3 cell line was derived. Since the metastatic potential of the C3 line declined over a number of years following repeated *in vitro* passages, a highly metastatic C3L5 line was derived by five cycles of repeated *in vivo* selections for spontaneous lung micrometastases following subcutaneous transplantation of C3 cells into C3H/HeJ mice.[25] The C3L5 cells used in the present study were grown from frozen stock and maintained in RPMI 1640 medium (GIBCO; Burlington, ON) supplemented with 5% fetal calf serum (GIBCO) and 1% penicillin-streptomycin (Mediatech; Washington, DC) in a humidified incubator, 5% CO_2.

In Vivo *Assay for Tumor-Induced Angiogenesis*

We devised a novel *in vivo* model of tumor angiogenesis based on the protocol of Kibbey et al.[26] These authors used conventional Matrigel, a reconstituted basement membrane, which is liquid at 4°C and forms a solid gelatinous mass at body temperature. Measurable angiogenesis occurred within these implants, possibly due to angiogenic growth factors present in the conventional Matrigel. In our application of the assay we used growth factor-reduced Matrigel, which, unlike conventional Matrigel, did not stimulate angiogenesis on its own. However, when tumor cells were suspended in growth factor-reduced Matrigel as a component of the subcutaneous implant, a strong angiogenic response was observed, which was easily and objectively quantifiable. Based on several pilot experiments in which the implant volume, tumor cell number, and implant duration were varied, we standardized the assay (the detailed kinetics of tumor-induced angiogenesis in these implants are not presented here). This assay was used to examine the effects of NO on the angiogenic response by administering L-NAME or its inactive enantiomer, D-NAME, to mice using osmotic minipumps (pilot experiments established an equivalent angiogenic response in animals receiving D-NAME and those receiving no treatment). The angiogenic response was evaluated by examining the gross morphology of the Matrigel implants and quantifying neovascularization in sections stained with Masson's trichrome or immunostained for the endothelial-specific CD31 antigen (PECAM). In addition, we documented the mass (weight in mg) of the implants on retrieval and systematically analyzed sections of tumor cell-inclusive implants for area quantification of three histologically distinct regions: peripheral tumor-free stromal tissue feeding blood vessels into the more deeply located tumor tissue; viable tumor tissue; and necrotic regions, to determine the effects of therapy on the various components of the implants.

In the inguinal region, mice received subcutaneous implants of 5×10^4 C3L5 cells suspended in growth factor reduced Matrigel (Collaborative Research, Bedford, MA) (3.5 mg of Matrigel in 0.5 ml of RPMI 1640), and on the contralateral side as controls, the equivalent amount of Matrigel alone. Immediately thereafter, osmotic minipumps (ALZA Corporation, Palo Alto, CA) were implanted subcutaneously, providing a constant systemic supply (0.5 ml/hour; 25 mg/200 μl 0.9% NaCl) of L-NAME to one group (n = 15), or D-NAME to the other group (n = 15) (both drugs purchased from Sigma Chemical Co., St. Louis, MO) for the experiment duration (14 days). This experiment was performed on two separate occasions; both experiments were conducted using the same protocol and sample size (ie, n = 15 animals/group).

Mice were sacrificed using an overdose of pentobarbital and the Matrigel implants were removed and divided in half; therefore paraffin and frozen sections were obtained from the same sample. Samples fixed in 4% paraformaldehyde, processed for paraffin embedding, and sectioned were stained with Masson's trichrome or immunostained for eNOS and iNOS proteins. Alternatively, samples frozen in OCT were sectioned and analyzed immunohistochemically for CD31. Both types of sections (ie, Masson's trichrome stained or CD31 immunostained) were scanned at low power for areas containing new blood vessels (researcher blind to experimental condition); these areas were systematically imaged at 160× magnification using Northern Exposure (Empix Imaging Inc.), and individual vessel counts for each field were documented using Mocha Image Analysis Software (Jandel Scientific) to identify fields of maximum blood vessel density (ie, "hot spots"). Subsequently, "hot spots" were statistically analyzed for between-group differences using two different approaches: the average (n = 15 animals/group) of the maximal number of blood in one field per animal, and the average (n = 15 animals/group) of the average of three fields of maximal blood vessel density (taken in descending order) per animal. Masson's trichrome-stained sections were also used to quantify histologically distinct regions within implants (ie, peripheral stromal, healthy tumor, and necrotic regions); entire cross sections of the implants were digitally imaged; areas were then quantified, and data expressed as the number of pixels, using Mocha Image Analysis Software (researcher blind to experimental condition).

Immunohistochemical Localization of CD31 Antigen (PECAM-1)

OCT-fixed samples were stored at −80°C until sectioned; samples were sectioned at 5 μm thickness and stored at −20°C before immunostaining (sections stored for maximum of 2 days at −20°C). Frozen sections were fixed in ice-cold methanol (5 minutes, −20°C). Endogenous peroxidase activity was blocked with methanol containing 3% H_2O_2 (30 minutes, room temperature) before application of blocking serum: normal mouse serum (Cedarlane Laboratories Limited, Hornby, ON) diluted in 1% bovine serum albumin (1:10; 1 hour at room temperature in humidified chamber). Sections were then incubated with primary antibody: purified rat anti-mouse CD31 monoclonal antibody (1:50; overnight at 4°C in humidified chamber; Cedarlane Laboratories) followed by secondary antibody: biotinylated mouse anti-rat IgG-2a monoclonal antibody (1:100; 1 hour at room temperature in humidified chamber; Caltag Laboratories, San Francisco, CA). Avidin-biotin complex (ABC) (Vector Laboratories, Inc., Burlingame, CA) was then applied (1 hour at room temperature), followed by diaminobenzidine chromogen (Sigma); sections were then lightly counterstained with Mayer's hemalum. Negative controls were incubated with the equivalent concentration of rat IgG-2a (Caltag Laboratories) in place of primary antibody.

Immunohistochemical Localization of eNOS and iNOS Antigens

Paraffin-embedded implants were sectioned at 7 μm thickness. Following deparaffinization and rehydration of sections, endogenous peroxidase activity was blocked

using methanol containing 3% H_2O_2 before application of blocking serum (normal horse serum, 1:10; 1 hour at room temperature). Sections were then incubated with primary antibody: mouse monoclonal anti-eNOS or mouse monoclonal anti-macrophage iNOS (1:80; overnight at 4°C, or 1:50; overnight at 4°C; Transduction Laboratories, Lexington, KY) for eNOS and iNOS localization, respectively. Secondary antibody (biotinylated horse anti-mouse, 1:200; 1 hour at room temperature) was then applied, followed by ABC (1 hour at room temperature) and diaminobenzidine chromogen. Sections were lightly counterstained with Mayer's hemalum.

Data Analysis

Data were analyzed using SAS v6.12 on a Unix mainframe computer, and treatment groups (ie, L-NAME and D-NAME) compared using one-way analysis of variance. In quantifying neovascular response for each treatment group (n = 15 mice/group), results were expressed as the mean of the maximum number of microvessels in a single field (160× magnification) and the mean number of microvessels in three fields of maximum blood vessel density (160× magnification). Data from the duplicate experiment (n = 15 mice/group) were analyzed in the same manner. A probability of 0.05 was used in determining statistical significance.

Results

Gross Morphology of Implants

Figure 1 shows the gross morphology of tumor-exclusive implants (Figure 1A) and tumor-inclusive implants obtained from L-NAME and D-NAME-treated (Figure 1, B and C, respectively) animals. Tumor-exclusive implants from L-NAME and D-NAME-treated animals were small, translucent, and avascular. Tumor-inclusive implants were larger, and implants obtained from L-NAME-treated animals were less vascular than those obtained from D-NAME-treated animals.

Histological Evaluation of Vascularity of Implants—Masson's Trichrome Staining

Figure 1 shows Masson's trichrome staining of tumor-exclusive Matrigel implant (Figure 1D) (sections of tumor cell-exclusive implants were identical for L-NAME and D-NAME-treated animals) and tumor-inclusive implants obtained from L-NAME (Figure 1E) and D-NAME-treated animals (Figure 1F). This method stains fibrous tissue and stroma bluish-green. Blood vessels containing red blood cells stand out because of bright red staining of red blood cells. Other cells (including tumor cells) show pink staining of cytoplasm and dark magenta colored nuclei. Tumor-exclusive Matrigel implants obtained from L-NAME and D-NAME-treated animals were avascular and contained a few fibroblasts. Tumor-inclusive implants obtained from both treatment groups consisted of three histologically distinct regions, shown in Figure 1G (implant obtained from L-NAME-treated animal): a peripheral zone of stroma (S) containing feeder blood vessels; healthy tumor areas (T); and more centrally located necrotic areas (N) infiltrated with leukocytes. Areas of highest microvascular count were best identified in the stroma of tumor-inclusive implants in Masson's trichrome-stained sections. The stroma of tumor-inclusive implants obtained from L-NAME-treated animals appeared thinner and less vascular relative to those obtained from D-NAME-treated animals.

Histological Evaluation of Vascularity of Implants—CD31 Immunostaining

Figure 2 shows immunohistochemical localization of CD31 antigen in implants obtained from D-NAME and L-NAME-treated animals (Figure 2, A and B, respectively). This method stains endothelial cells brown and correctly identifies cells lining the microvasculature within the tumor component of the implants (in contrast to Masson's trichrome-stained sections, which predominantly identifies blood vessels within stromal areas). Nuclei are lightly counterstained with Mayer's hemalum. Neovascularization was reduced in tumor-inclusive implants obtained from L-NAME-treated animals relative to those obtained from D-NAME-treated animals.

Histological Evaluation of Implants—eNOS and iNOS Immunostaining

Immunohistochemical localization of eNOS antigen in tumor-inclusive implant and a negative control are shown in Figure 2, C and D, respectively. eNOS expression was observed in endothelial cells of the tumor vasculature (Figure 2C, inset), and a high proportion of tumor cells within implants obtained from animals treated with either L-NAME or D-NAME. Immunohistochemical localization of iNOS antigen is shown in Figure 2, E and F. Regardless of treatment group, tumor cells within the implants did not stain positively for iNOS. However, positive immunoreactivity for iNOS protein was observed in a significant proportion of macrophages located in peripheral stroma (Figure 2E), in the healthy tumor bordering the necrotic area (Figure 2F), and within the central necrotic area (not shown in Figure 2). Endothelial cells also stained positively for iNOS (Figure 2E, small arrow).

Quantification of Tumor-Induced Neovascularization—Masson's Trichrome Staining and CD31 Immunostaining

Figure 3 shows the number of blood vessels per unit area for Masson's trichrome-stained and CD31 immunostained sections; data are expressed as the maximum number of blood vessels per field and as the average number of blood vessels in three fields of maximal density. Irrespective of staining protocol or method of quan-

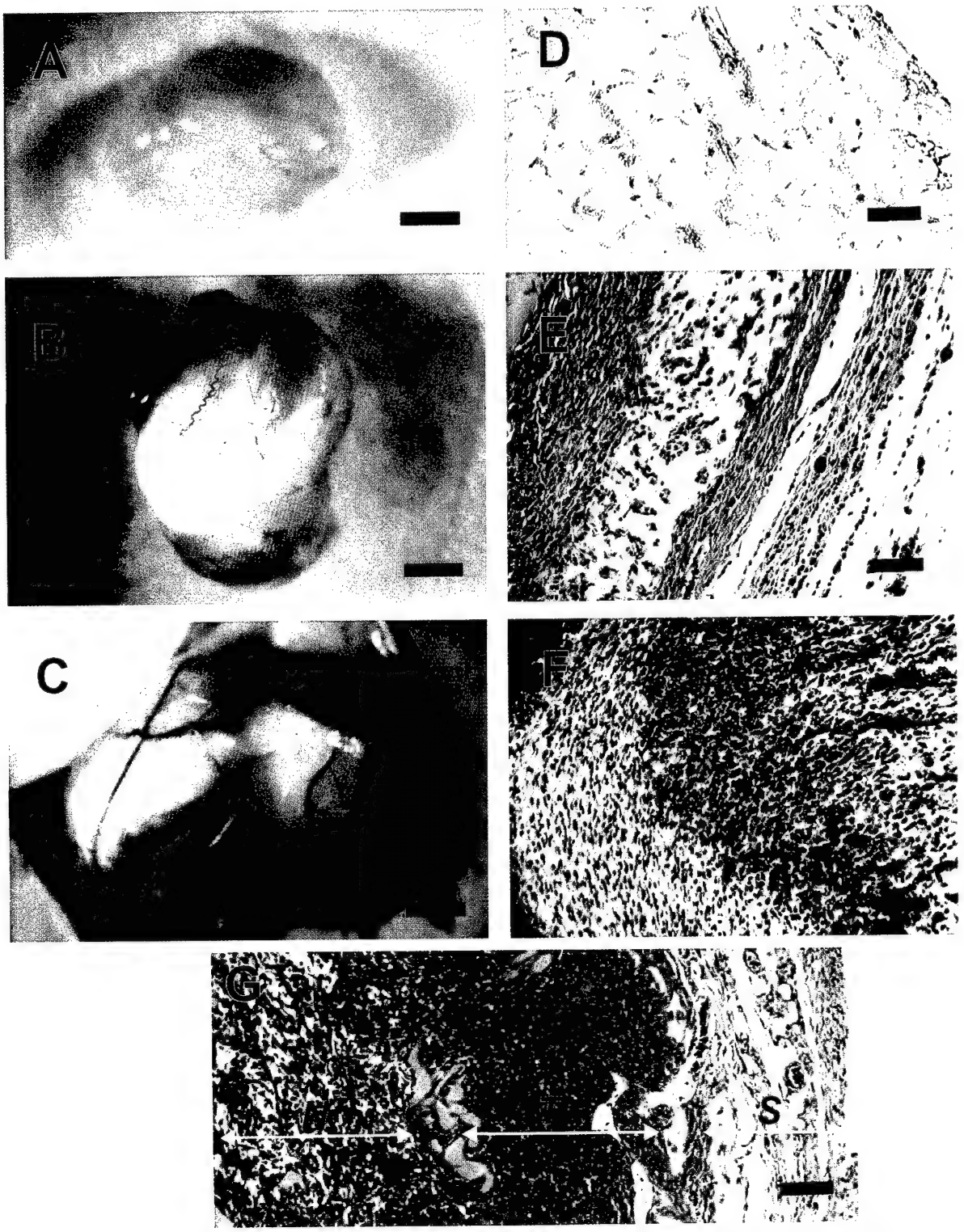
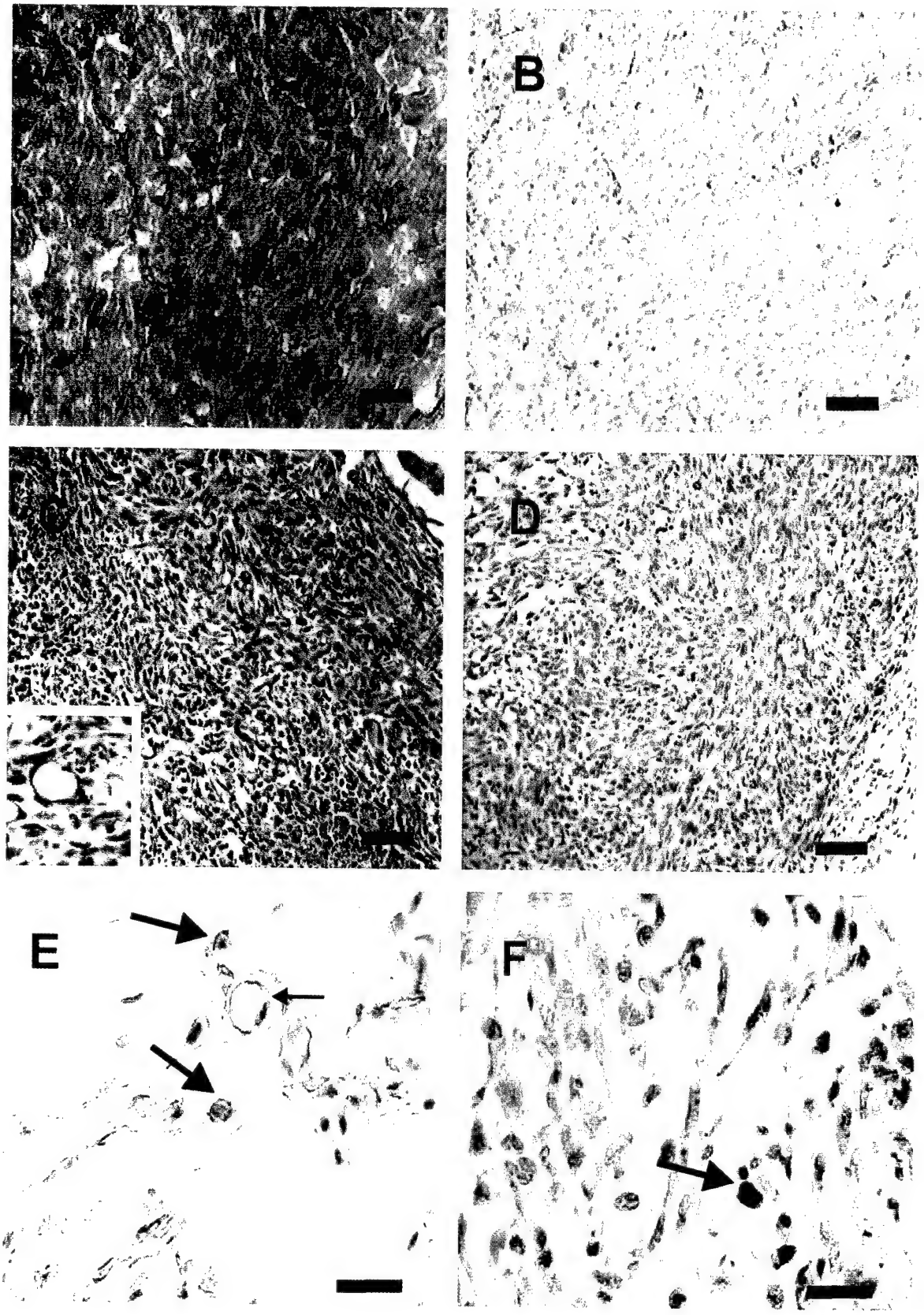


Figure 1. Gross morphology is shown for tumor-exclusive Matrigel implants (**A**) and tumor-inclusive Matrigel implants obtained from L-NAME- and D-NAME-treated animals (**B** and **C**, respectively). Tumor-exclusive implants were translucent, avascular, and unaffected by L-NAME or D-NAME treatment. Tumor-inclusive implants were larger, and those obtained from L-NAME-treated animals were less vascular relative to those obtained from D-NAME-treated animals. Photomicrographs of Masson's trichrome staining are shown for tumor-exclusive (**D**) and tumor-inclusive Matrigel implants obtained from L-NAME-treated (**E**) and D-NAME-treated (**F**) animals. Tumor-exclusive implants obtained from L-NAME- and D-NAME-treated animals were avascular and contained a few fibroblasts. The sections of tumor-inclusive implants in both treatment groups showed three histologically distinct areas, shown in **G** (from L-NAME-treated animal): peripheral stroma (S), adjacent tumor-dominant area (T), and central zone of necrosis (N). The stromal components in L-NAME-treated animals appeared thinner and less vascular relative to those in D-NAME-treated animals. Scale bars: **A–C**, 1 mm; **D–G**, 30 μm.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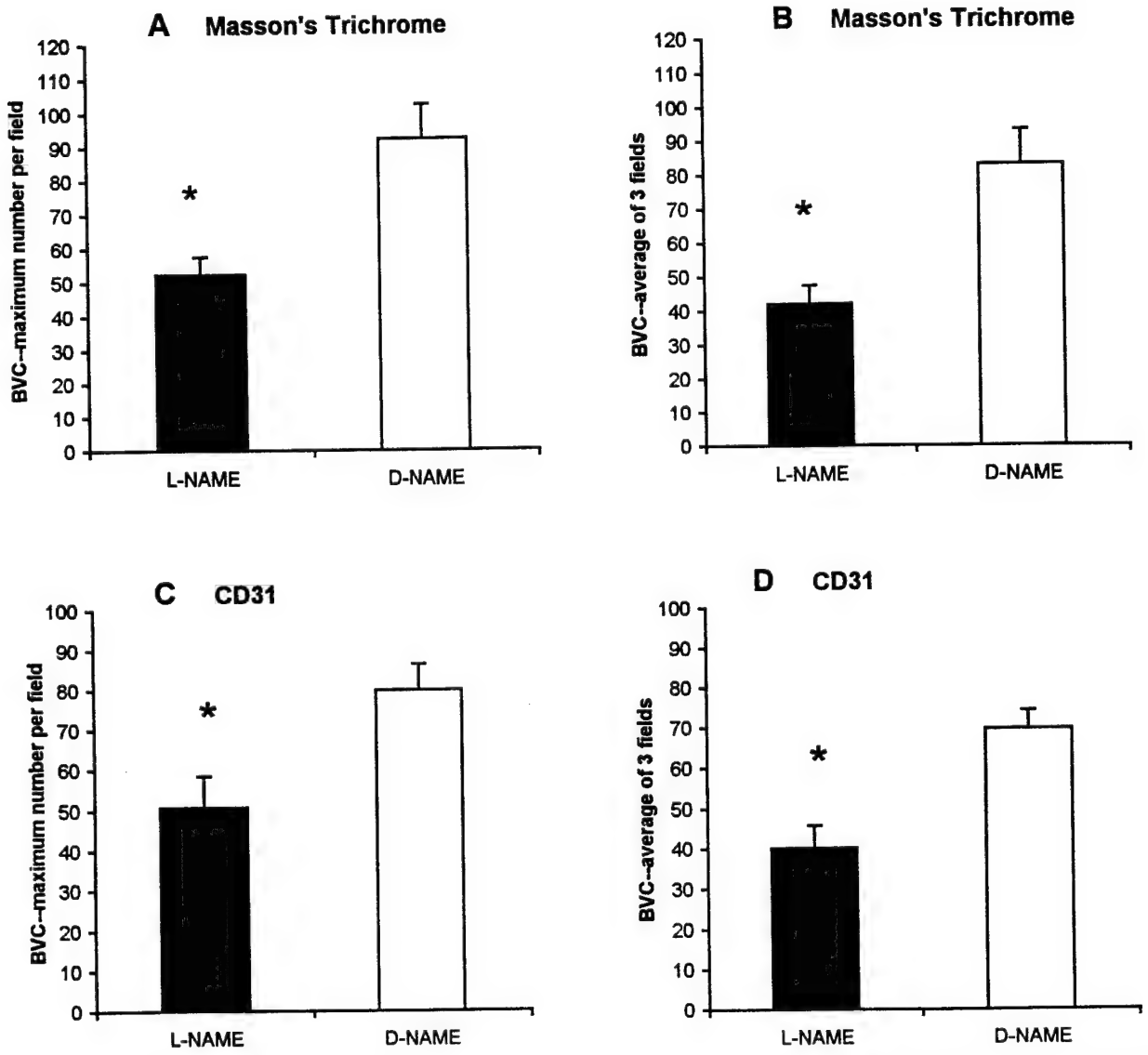

Figure 3. Quantification of tumor-induced neovascularization in sections stained with Masson's trichrome (A and B) and immunostained for CD31 (C and D). The data were expressed as the maximum number of blood vessels per field (mean ± SE; $n = 15$ animals/group) and as the average number of blood vessels in 3 fields of maximal density (mean ± SE; $n = 15$ animals/group). Irrespective of staining protocol or method of quantification used, the neovascular response was reduced in L-NAME-treated mice relative to those treated with D-NAME as indicated by the P value in each panel: A, $P < 0.003$; B, $P < 0.001$; C, $P < 0.0099$; D, $P < 0.0009$. BVC, blood vessel count.

tification used, the neovascular response was reduced in L-NAME-treated mice relative to those treated with D-NAME. Tumor-induced neovascularization, measured by Masson's trichrome, was reduced in implants obtained from L-NAME-treated animals when data were expressed as 1) the maximum number of blood vessels per field (L-NAME, 52.3 ± 5.9; D-NAME, 92.3 ± 11.3, $P < 0.003$) and 2) as the average number of blood vessels in three fields (L-NAME, 42.1 ± 5.4; D-NAME, 83.2 ± 10.2, $P < 0.001$) (Figure 3, A and B, respectively). L-NAME treatment reduced tumor-induced neovascularization, as measured by CD31 immunostaining, when data were expressed 1) as the maximum number of blood vessels per field (L-NAME, 50.8 ± 7.6; D-NAME, 79.9 ± 6.5, $P < 0.0099$) and 2) as the average number of blood vessels in three fields (L-NAME, 40.1 ± 5.6; D-NAME, 69.6 ± 4.8,

Figure 2. Immunohistochemical localization of CD31 antigen is shown at **top** in tumor-inclusive implants from D-NAME- and L-NAME-treated animals (**A** and **B**, respectively), identifying endothelial cells lining the microvasculature within the tumor component of the implants. Neovascularization was reduced in L-NAME-treated animals relative to those treated with D-NAME. Immunohistochemical localization of eNOS antigen is shown at **center** in tumor-inclusive implants; positive immunostaining and negative control are shown (**C** and **D**, respectively). A high proportion of tumor cells and endothelial cells lining the tumor vasculature (**inset** in **C**) within implants expressed eNOS, regardless of treatment group. The **bottom** shows immunohistochemical localization of iNOS antigen in peripheral stroma (**E**) and healthy tumor (**F**) bordering necrotic area. Expression of iNOS did not differ between treatment groups. Positive immunoreactivity for iNOS protein was evident in macrophages (**large arrows**) located in stromal (**E**) and tumor (**F**), as well as within central necrotic (not shown) areas of the implants in both treatment groups. Endothelial cells (**small arrow**) stained positively for iNOS, and some nonspecific staining of stromal tissue was observed in both treatment groups. Scale bars: **A–D**, 30 μm; **E–F**, 50 μm; **C, inset**, 60 μm.

$P < 0.0009$) (Figure 3, C and D, respectively). Results obtained from the duplicate experiment were similar and are not presented.

Quantification of Histologically Distinct Areas within Implants

Quantification of the various tissue compartments contained within tumor cell-inclusive implants indicated that the quantity of peripherally located stromal tissue was reduced in L-NAME relative to D-NAME-treated animals (L-NAME, 4998.5 ± 1055.2 pixels; D-NAME, 9758.3 ± 1515.4 pixels, $P < 0.02$), and the quantity of necrotic tissue was higher in L-NAME relative to D-NAME-treated animals (L-NAME, 73709.5 ± 8638.0 pixels; D-NAME, 38434.5 ± 7918.3 pixels, $P < 0.007$). In addition, the mass of viable tissue (ie, stroma and tumor cells), was lower in L-NAME relative to D-NAME-treated animals (L-NAME, 17.1 ± 3.5%; D-NAME, 28.8 ± 4.0%, $P < 0.04$). Values for the mass of viable tissue were calculated using the following formula: weight of implant (mg) × (1 − necrotic fraction of the implant).

Discussion

Angiogenesis, the development of new blood vessels from the pre-existing vascular bed, is an essential feature of many physiological conditions including wound healing, embryonic development, and endometrial proliferation. Numerous pathological conditions, such as diabetic retinopathy, rheumatoid arthritis, and tumor growth are also characterized by abnormal neovascularization. Growth of solid tumors cannot proceed beyond a microscopic size without the development of an extensive vascular system.[27] Furthermore, because the degree of vascularization often correlates with poor clinical prognosis and increased likelihood of metastasis of a number of human tumors,[28,29] targeting angiogenesis in the therapeutic intervention of cancer has received substantial attention. Although a number of compounds characterized as inhibitors of angiogenesis have entered clinical trials, intense efforts to identify potent angiogenesis inhibitors with improved selectivity continue. However, a consistent limitation of these investigations has been the availability of simple, reproducible, reliable, and easily quantifiable assays that reflect *in vivo* systems for tumor-induced angiogenesis. Overly simplistic cellular *in vitro* systems and technical difficulties of currently used *in vivo* angiogenesis assays are important limiting factors.

In vitro models of angiogenesis[22,30] may not be ideal for measuring tumor-induced angiogenesis because of the inability in providing all of the necessary cells and/or factors that may interact in the *in vivo* tumor environment. The most widely used *in vivo* systems are the rabbit cornea and the chick chorioallantoic membrane (CAM) assays, in which variability of results and subjectivity in quantification remain important limitations.[31] A major concern of the rabbit cornea assay is the potential development of xenograft reactions after tumor implantation. Although the cornea is an immunoprivileged site, as it gradually becomes vascularized, the possible contribution of xenograft reactions to the angiogenic response cannot be disregarded. The CAM assay does not present this problem, because the host is naturally immunodeficient. However, major disadvantages of this assay are the time limit of 7 to 10 days imposed by embryo growth and acquisition of immunocompetence[32] and difficulties in objectively quantifying the neovascular response.[31,33] Another potential concern is inherent with properties of the chick chorioallantoic membrane, which is a growing and developing embryonic structure; the relative contribution of vasculogenesis and/or angiogenesis, two discrete processes which are differentially regulated, to the development of new blood vessels may not be clear.

The present *in vivo* model of tumor-induced angiogenesis is devoid of these limitations. The assay is simple, reproducible, and objectively quantifiable. Suspension of tumor cells within the Matrigel matrix serves to effectively immobilize tumor cells; the ensuing angiogenic response is organized, and all stages of the neovascular response can be quantified. Therefore, the kinetics of tumor development and neovascularization may be followed for a considerable length of time (eg, 2 weeks in the present experiment); in separate experiments, we have established that the experimental procedure can be extended (eg, up to 6 weeks). Furthermore, because the developing blood vessels converge within a discrete area, retrieval and quantification of developing blood vessels are simple and complete. Use of a specific inoculation site in the murine model minimizes variation of the angiogenic response. Although we used an inbred mouse strain and its syngeneic tumor, nude mice could appropriately serve as the host for xenografts in other applications of this assay, and in particular, for human tumors.

We used the present assay to evaluate the effects of NO on the angiogenic response by administering L-NAME or D-NAME to mice using osmotic minipumps in two separate experiments. Results from both clearly showed that, irrespective of method of quantification, NOS inhibition dramatically reduced the neovascular response. The growth patterns of histologically distinct areas present within the implants were differentially affected by NOS inhibition. The stromal component of the implants, which supports the vascular supply, was reduced in L-NAME- relative to D-NAME-treated animals. In addition, there was increased necrosis and reduced viable tissue mass within implants obtained from L-NAME relative to D-NAME-treated animals, supporting prior observations of antitumor effects of NOS inhibition in mice transplanted with C3L5 mammary adenocarcinomas.[14,15] Therefore, the antitumor and antimetastatic effects of NOS inhibition, previously attributed in part to reduced tumor cell invasiveness,[16] may also be explained by reduced neovascularization.

Inherent NOS activity of the eNOS-expressing mammary adenocarcinoma cells used in the present research is likely the major source of NO contributing to the NO-mediated induction of angiogenesis. eNOS expression

by endothelial cells, as well as iNOS expression by some macrophages and endothelial cells, may serve as additional minor sources of NO in these tumor implants.

The precise molecular mechanisms responsible for reduced angiogenesis with NOS inhibition in our model remain to be determined. NO is required for endothelial cell proliferation, migration, and organization, key components of the angiogenic cascade.[22,34,35] Using a rabbit cornea assay, it has been shown that NO is a downstream mediator of vascular endothelial growth factor (VEGF)-induced angiogenesis, since it could be blocked by administering L-NAME.[35] Further evidence supports this notion; angiogenesis in response to tissue ischemia (an inducer of VEGF) was reduced in eNOS −/− mice.[36] VEGF-stimulated proliferation of endothelial cells, triggered by NO, was shown to require intracellular signaling via cGMP-dependent protein kinase,[37] Raf-1 kinase,[37] and mitogen- activated protein kinase.[35] Our preliminary results (data not shown) indicate that the C3L5 mammary adenocarcinoma cells used in the present research express VEGF protein *in vitro*. Whether VEGF expression in these cells is induced by endogenous NO remains to be determined; an up-regulation of VEGF mRNA by NO was reported for rat mesangial cells.[38] Co-expression of eNOS and VEGF in C3L5 cells may equip them with a dual advantage in inducing NO-mediated angiogenesis from the host vasculature, VEGF-mediated stimulation of NO in the vascular endothelium and NO produced by tumor cells by activation of eNOS.

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NITRIC OXIDE PROMOTES MURINE MAMMARY TUMOUR GROWTH AND METASTASIS BY STIMULATING TUMOUR CELL MIGRATION, INVASIVENESS AND ANGIOGENESIS

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The contributory role of nitric oxide (NO) on tumour growth and metastasis was evaluated in a murine mammary tumour model. NO synthase (NOS) protein expression levels were examined in spontaneously arising C3H/HeJ mammary adenocarcinomas and respective lung metastases. In addition, 2 clonal derivatives of a single spontaneous tumour differing in metastatic phenotype (C3L5 and C10; highly and weakly metastatic, respectively) were utilised to investigate *(i)* the relationship between NOS expression levels and the biological behaviour of tumour cells (e.g., *in vitro* migratory and invasive capacities, *in vivo* tumour growth rate and metastatic and angiogenic capacities) and *(ii)* whether tumour-derived NO stimulated the invasive, migratory and angiogenic capacities of tumour cells. A heterogeneous pattern of endothelial NOS (eNOS) expression was observed in tumour cells in spontaneous primary tumours, and eNOS expression was higher in undifferentiated relative to differentiated tumour zones. However, tumour cells in lung metastatic sites were always strongly eNOS-positive, suggesting that eNOS expression facilitated metastasis. Findings using clonal derivatives supported this notion; s.c. primary tumour growth rate, efficiency of spontaneous metastasis and eNOS expression were higher for C3L5 relative to C10 cell lines. Nevertheless, lung metastases derived from both tumour cell lines were always strongly and homogeneously eNOS-positive. C3L5 cells were more invasive than C10 cells *in vitro,* but the migratory capacities of the cell lines did not differ. However, migration and invasiveness of both cell lines were inhibited with L-NAME and restored with excess L-arginine. Tumour-associated angiogenesis, measured in Matrigel implants inclusive of tumour cells, was higher for C3L5 relative to C10 cells, and C3L5-induced angiogenesis was reduced with chronic L-NAME treatment of host animals. These findings suggest that tumour-derived eNOS promoted tumour growth and metastasis by multiple mechanisms: stimulation of tumour cell migration, invasiveness and angiogenesis. *Int. J. Cancer* 86:30–39, 2000.
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Nitric oxide (NO), an inorganic free radical gas, is synthesised from the amino acid L-arginine by a group of enzymes, the nitric oxide synthases (NOS). Three isoforms of the enzyme have been identified: endothelial (e) and neuronal (n) isoforms are Ca^{2+}/calmodulin-dependent and constitutively expressed. The inducible isoform (iNOS) is Ca^{2+}/calmodulin-independent and induced in the presence of inflammatory cytokines or bacterial products. When constitutively expressed, NO produced at low levels is an important mediator of physiological functions such as vasodilation, inhibition of platelet aggregation and neurotransmission. Under inductive conditions, high levels of NO can mediate antibacterial and anti-tumour functions; however, sustained, chronically produced NO contributes to many pathological conditions, including inflammation and cancer (reviewed by Moncada and Higgs, 1993; Knowles and Moncada, 1994).

The role of NO in tumour biology has been extensively studied; overall, an overwhelming amount of evidence suggests a positive association between NO and tumour progression (Lala and Orucevic, 1998; Thomsen and Miles, 1998). Over-expression of NOS enzymes and/or NOS activity has been positively correlated with the degree of malignancy in the human reproductive tract (*i.e.,* ovarian, uterine) cancers (Thomsen *et al.,* 1994), CNS tumours (Cobbs *et al.,* 1995) and mammary tumours (Thomsen *et al.,* 1995; Dueñas-Gonzalez *et al.,* 1997). iNOS has been detected in stromal elements and eNOS in tumour vasculature in a majority of gastric carcinomas (Thomsen and Miles, 1998); relative to benign prostatic hyperplasia, iNOS expression was higher in prostatic carcinomas (Klotz *et al.,* 1998). Total NOS activity is increased in carcinomas of the larynx, oropharynx, oral cavity (Gallo *et al.,* 1998) and adenocarcinomas of the lung (Fujimoto *et al.,* 1997) relative to normal healthy control tissue.

Experimental tumour models have provided direct evidence for a promoting role of NO in tumour progression. Treatment with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) reduced NO production and tumour growth in a rat adenocarcinoma model (Kennovin *et al.,* 1994). Induction of iNOS with lipopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine mammary tumour cells stimulates tumour growth and metastasis *in vivo* (Edwards *et al.,* 1996). Furthermore, iNOS transduction in a human colon adenocarcinoma line results in enhanced tumour growth and vascularity when transplanted in nude mice (Jenkins *et al.,* 1995). We have studied the role of NO in tumour progression/metastasis using a murine mammary tumour model which includes spontaneously arising mammary adenocarcinomas and 2 clonal derivatives of a spontaneous tumour which differ in metastatic capacity. Preliminary studies of spontaneously arising mammary tumours in C3H/HeJ retired breeder female mice revealed that cells in primary tumours were distinctly heterogeneous in eNOS protein expression. However, a strong and homogeneous expression pattern was observed at metastatic lung sites, suggesting that eNOS expression provides a selective advantage to metastasis (Lala and Orucevic, 1998). Further evidence supported this notion. A highly metastatic cell line, C3L5, clonally derived from a spontaneously arising mammary tumour, strongly expressed eNOS protein *in vitro* and *in vivo* and iNOS protein upon stimulation with LPS and IFN-γ (Orucevic *et al.,* 1999). Treatment of C3L5 mammary tumour-transplanted animals with the NOS inhibitors N^G-methyl-L-arginine (L-NMMA) or L-NAME reduced both primary tumour growth and spontaneous lung metastases (Orucevic and Lala, 1996*a,b*; Lala and Orucevic, 1998). The present study used this murine mammary tumour model to further examine the contributory role of NO in tumour progression and metastasis and the underlying mechanisms. A large number of spontaneous mammary tumours and respective lung metastases were utilised to examine the relationship between levels of NOS protein expression at primary tumour sites and the degree of morphological differentiation of tumour cells and tumour growth rates and to compare the levels of NOS expression between primary and metastatic lesions. Clonally derived C3L5 (highly metastatic) and C10 (weakly metastatic) tumour cell lines were utilized to examine *(i)* levels of NOS protein expression *in vitro* and *in vivo* (at primary and metastatic tumour sites), *(ii)* whether the levels of NOS protein

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expression and NO production by tumour cells were correlated with invasive or migratory abilities *in vitro* or to their biological behaviour *in vivo* (*i.e.,* tumour growth rate, capacities for metastases formation and angiogenesis) and *(iii)* whether NO production by tumour cells was causally related to invasion, migration and angiogenesis.

MATERIAL AND METHODS

Mice

Female C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME); retired breeder mice (approx. 6 months old) were utilised in spontaneous mammary tumour experiments, and 6- to 8-week-old mice were used in experiments related to tumour transplantation and angiogenesis with C3L5 and C10 cells. Upon arrival, animals were randomised into treatment groups; experimental procedures began after a 1-week acclimatization period. Throughout the investigation, animals had free access to food (standard mouse chow) and water and were maintained on a 12 hr light/dark cycle. Animals were treated in accordance with guidelines set by the Canadian Council on Animal Care.

Spontaneous mammary tumour development

Fifty C3H/HeJ female retired breeder mice were monitored 2 times per week for spontaneous mammary tumour development (20 tumours studied) as previously reported (Lala *et al.,* 1997). After initial localisation of a primary tumour, tumour growth rate was monitored daily; minimum and maximum diameters were measured using digital calipers, and tumour volume was calculated using the equation tumour volume $= 0.52a^2b$, where a and b are the minimum and maximum tumour diameters, respectively (Baguley *et al.,* 1989). When tumours had grown for 8 to 12 weeks, mice were killed using an overdose of pentobarbital and primary tumours and lungs inclusive of metastatic foci were removed, processed for paraffin embedding and immunostained for eNOS and iNOS antigens.

Tumour cell lines

Two murine mammary adenocarcinoma cell lines were utilised: C3L5, a highly metastatic line, and C10, a weakly metastatic line. Both were originally derived from a spontaneous tumour that developed in a C3H/HeJ female retired breeder mouse. Cells from the primary tumour (T58) demonstrated moderate metastatic capacity in early *in vitro* passages (Brodt *et al.,* 1985). Two clones were then derived from T58 cells: the weakly metastatic C10 line currently used and C3, a highly metastatic line. Since the metastatic capacity of the C3 line declined after several years of repeated *in vitro* passages (Lala *et al.,* 1986), a highly metastatic C3L5 line was derived by 5 cycles of repeated *in vivo* selections for spontaneous lung micrometastases following s.c. transplantation of C3 cells into C3H/HeJ mice and retransplantation of dispersed lung micrometastases s.c. in syngeneic mice. This led to production of the highly metastatic cell line C3L5 (Lala and Parhar, 1993), which has since maintained its strong metastatic phenotype. C3L5 and C10 cells were grown from frozen stock and maintained in RPMI-1640 medium (GIBCO, Burlington, Canada) supplemented with 5% FCS (GIBCO) and 1% penicillin–streptomycin (Mediatech, Washington, DC) in a humidified incubator with 5% CO_2.

Tumour transplantation studies

C3L5 and C10 cells, grown in monolayer, were harvested by brief exposure to 0.05% trypsin–PBS–EDTA solution. C3L5 or C10 cells (5×10^5), suspended in 0.5 ml of RPMI, were injected s.c. in the mammary line in the left axillary region of 6- to 8-week-old C3H/HeJ female mice (n = 15 mice/group). Trypan blue exclusion staining ensured adequate tumour cell viability (*i.e.,* >95%). Tumour growth was monitored using digital calipers; 2 times per week, the minimum and maximum diameters were recorded and tumor volume was calculated as described for spontaneous tumours. Twenty-one days after tumour transplantation, mice were killed using an overdose of pentobarbital and primary tumours removed, fixed in 4% paraformaldehyde and processed for paraffin embedding. Lungs were inflated *in situ* with Bouin's fixative, removed and assessed for lung surface colonies using a dissecting microscope (experimenter blind to experimental condition). Immunostaining of both primary tumours and the corresponding lung metastases for eNOS and iNOS protein was conducted as described below.

Immuno-cytochemical detection of NOS enzymes in cells propagated in vitro

C3L5 and C10 cells were grown in complete RPMI-1640 medium alone or in complete medium containing IFN-γ (1,000 U/ml; GIBCO BRL, Grand Island, NY) and LPS (100 ng/ml; Sigma, St. Louis, MO) for 24 hr on chamber slides (Nunc, Naperville, IL) in a humidified incubator (37°C, 5% CO_2). Cells were fixed in ice-cold methanol (–20°C, 5 min). Endogenous peroxidase activity was blocked with methanol containing 3% H_2O_2 (room temperature, 5 min), and cell membranes were permeabilized using 0.25% Triton X-100 in 0.2% BSA in PBS prior to application of blocking antibody: normal horse serum diluted in 0.2% BSA (1:10; 1 hr at room temperature in a humidified chamber). Cells were then incubated with primary antibody: mouse monoclonal antibody (MAb) anti-eNOS or mouse anti-macrophage iNOS MAb (1:80 diluted in 0.2% BSA overnight at 4°C or 1:50 diluted in 0.2% BSA overnight at 4°C; Transduction Laboratories, Lexington, KY) for eNOS and iNOS localisation, respectively. Secondary antibody, biotinylated horse anti-mouse (1:200 diluted in 0.2% BSA, 1 hr at room temperature), was then applied, followed by avidin–biotin complex (Vector, Burlingame, CA) (1 hr at room temperature) and DAB chromogen (Sigma). Negative controls were incubated with the equivalent concentration of mouse IgG (Dako, Horsholm, Denmark) in place of primary antibody. Immuno-cytochemical staining of human umbilical vein endothelial cells (HUVECs) for eNOS served as positive controls.

Immuno-histochemical detection of NOS enzymes in spontaneous and transplanted tumours

Paraformaldehyde-fixed, paraffin-embedded primary tumours and lungs inclusive of metastatic foci were sectioned at 7 μm thickness. Following deparaffinization and rehydration of sections, endogenous peroxidase activity was blocked using methanol containing 3% H_2O_2 prior to application of blocking serum and primary and secondary antibodies, as described above. Sections were lightly counterstained with Mayer's haemalum, and those sections used to quantify immunostaining intensities were incubated with metal-enhanced DAB (Pierce, Rockford, IL) and not counterstained.

Quantitative analysis of immuno-histochemical staining in tissue sections

The intensity of immuno-histochemical staining was quantified using a method similar to that of Lehr *et al.* (1997). Digitized images of non-counterstained primary tumour sections and lung metastases were obtained and imported into the image analysis software program Mocha (Jandel, San Rafael, CA); pixels of the black and white images were inverted and remapped (*i.e.,* black pixels converted to white and white converted to black); therefore, absolute white was measured as 0 and absolute black as 255 grey level units. The average intensity of immuno-histochemical staining in healthy (non-necrotic) tumour tissue was quantified and compared between experimental groups (*i.e.,* C3L5 and C10) and corresponding negative control sections.

In vitro *invasion and migration assays*

Both invasion and migration assays were conducted in transwells fitted with millipore membranes (6.5 mm filters, 8 μm pore size; Costar, Toronto, Canada). In the invasion assay, cells degraded and passed through a Matrigel barrier prior to migrating through membrane pores. Thus, in the invasion assay,

membranes were coated with 120 μl growth factor–reduced Matrigel (1:20 dilution in RPMI-1640 medium; Collaborative Research, Bedford, MA). For both assays, 2.5×10^4 C3L5 or C10 cells/100 μl complete RPMI were plated in upper wells of transwell chambers containing either 200 μl complete RPMI, complete RPMI and L-NAME (0.01, 0.1 or 1 mM; Sigma) or complete RPMI, L-NAME and excess L-arginine (5 mM, Sigma) (total volume in upper chamber 200 μl). Bottom wells contained 800 μl complete RPMI. Chambers were gently shaken for 1 hr at room temperature, followed by 24, 48 or 72 hr incubation (37°C, 5% CO_2). After incubation, cells from the upper surface of millipore membranes were completely removed with gentle swabbing and remaining migrant cells were fixed and stained using the Diff-Quik Stain Set (Dada, Düdingen, Switzerland). Membranes were then rinsed with distilled H_2O, gently cut from transwells and mounted onto glass slides. Cellular invasion and migration indices were determined by counting the number of stained cells on membranes in 5 randomly selected, non-overlapping fields at 400× magnification under a light microscope (researcher blind to experimental condition).

Assay for in vitro *NO production*

C3L5 and C10 cell culture media were collected at the same time points and, in conditions identical with those used in invasion and migration assays, stored at –20°C until assayed. Levels of NO were measured by determining levels of inorganic NO_2–, a stable product of oxidized NO (Moncada and Higgs, 1993), in the Greiss reaction (Green *et al.,* 1982), using a procedure previously established in this laboratory (Orucevic and Lala, 1996a). Briefly, samples of culture medium were diluted in de-ionized H_2O (1:1) and proteins precipitated using 50 μl 30% $ZnSO_4$ and 1 ml of dilute sample, followed by centrifugation (8,000 *g,* 5 min); 1 ml of supernatant was incubated (room temperature, 30 min) with 300 μl 0.5 M ammonium chloride, 100 μl 0.06 M sodium borate and 50 mg acid-washed cadmium filings (Davison and Woof, 1978). The mixture was centrifuged (400 *g,* 7 min), and 1 ml of supernatant was added to Greiss reagent and incubated (room temperature, 10 min). Greiss reagent was prepared by mixing equal parts 1% sulphanilic acid (Sigma) and 0.1% naphthylethylenediamine (Sigma) in 2% phosphoric acid. Absorbance of samples at 543 nm was measured using a spectrophotometer; concentration of NO_2– in media samples was determined from the sodium nitrite standard curve, which was linear for 0 to 100 μM of nitrite.

In vivo *tumour-induced angiogenesis assay*

Levels of tumour-induced angiogenesis *in vivo* were quantified using a novel assay devised in our laboratory and based on our observation that angiogenesis was induced in s.c. implants of growth factor–reduced Matrigel only when tumour cells were suspended in the Matrigel (Jadeski and Lala, 1999). We compared angiogenesis induced by C3L5 and C10 cells and examined the effects NOS inhibition by chronically administering L-NAME or its inactive enantiomer, D-NAME, to implant-bearing mice using osmotic minipumps.

In the inguinal region, mice received s.c. implants of 5×10^4 C3L5 or C10 cells suspended in growth factor–reduced Matrigel (3.5 mg Matrigel in 0.5 ml RPMI) and, on the contralateral side as controls, the equivalent amount of Matrigel alone. Immediately thereafter, osmotic minipumps (ALZA, Palo Alto, CA) were implanted s.c., providing a constant systemic supply (0.5 μl/hr, 25 mg/200 μl 0.9% NaCl) of L-NAME (n = 15/group) or D-NAME (n = 15/group) (both drugs purchased from Sigma) for the duration of the experiment (14 days).

Mice were killed using an overdose of pentobarbital and Matrigel implants removed, fixed in 4% paraformaldehyde, processed for paraffin embedding, sectioned and stained with Masson trichrome. Sections were scanned at low power for areas containing new blood vessels (researcher blind to experimental condition); these areas were systematically imaged at 160× magnification using Northern Exposure (Empix Imaging, Mississauga, ON) and individual vessel counts for each field documented using Mocha Image Analysis Software (Jandel) to identify fields of maximum blood vessel density (*i.e.,* hot spots). Subsequently, hot spots were statistically analysed for between-group differences by determining the average (n = 15 animals/group) of 3 fields of maximal blood vessel density (taken in descending order) per animal.

Statistical analysis

Data were analysed using the SAS (Cary, NC) system for Windows, release 6.12. Data comparing 2 means were tested using Student's *t*-test; those comparing multiple (*i.e.,* more than 2)

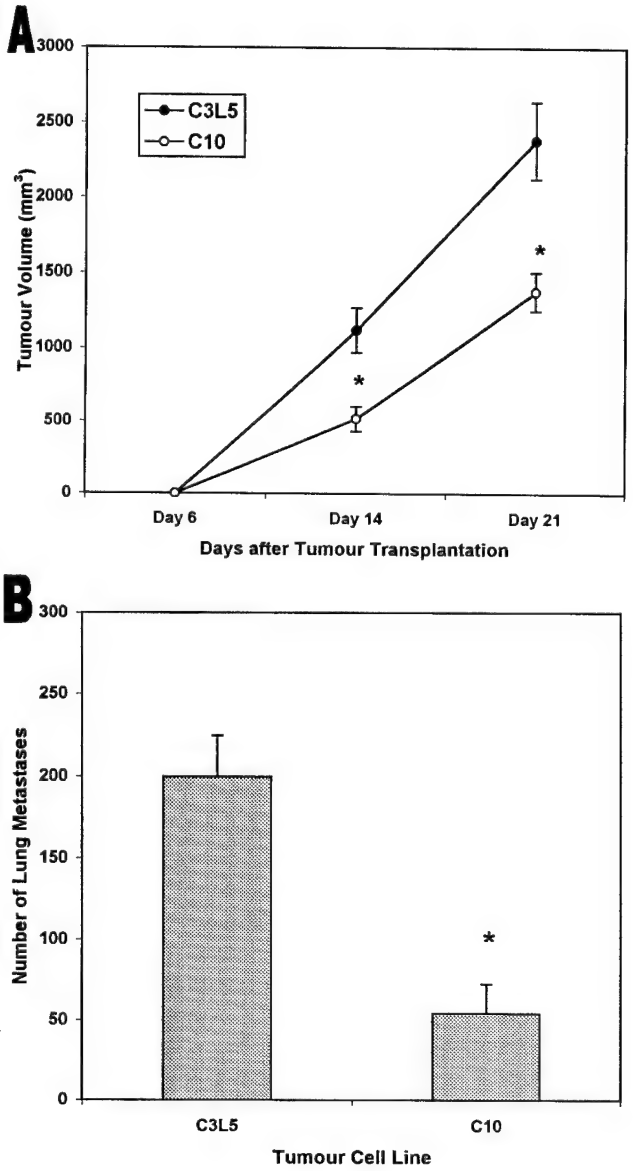


FIGURE 1 – Primary tumour growth rate and metastatic lung colony formation in C3H/HeJ mice after s.c. injection of 5×10^5 C3L5 or C10 tumour cells. *(a)* At 14 and 21 days after tumour transplantation, primary tumour volumes were significantly lower ($*p < 0.0001$) for C10 than for C3L5 transplants. Data represent means ± SE (n = 15/group). *(b)* Mean number of metastatic lung nodules 3 weeks after tumour transplantation. Fewer lung colonies formed in mice bearing C10 than C3L5 tumour transplants ($*p < 0.001$). Data represent means ± SE (n = 15/group).

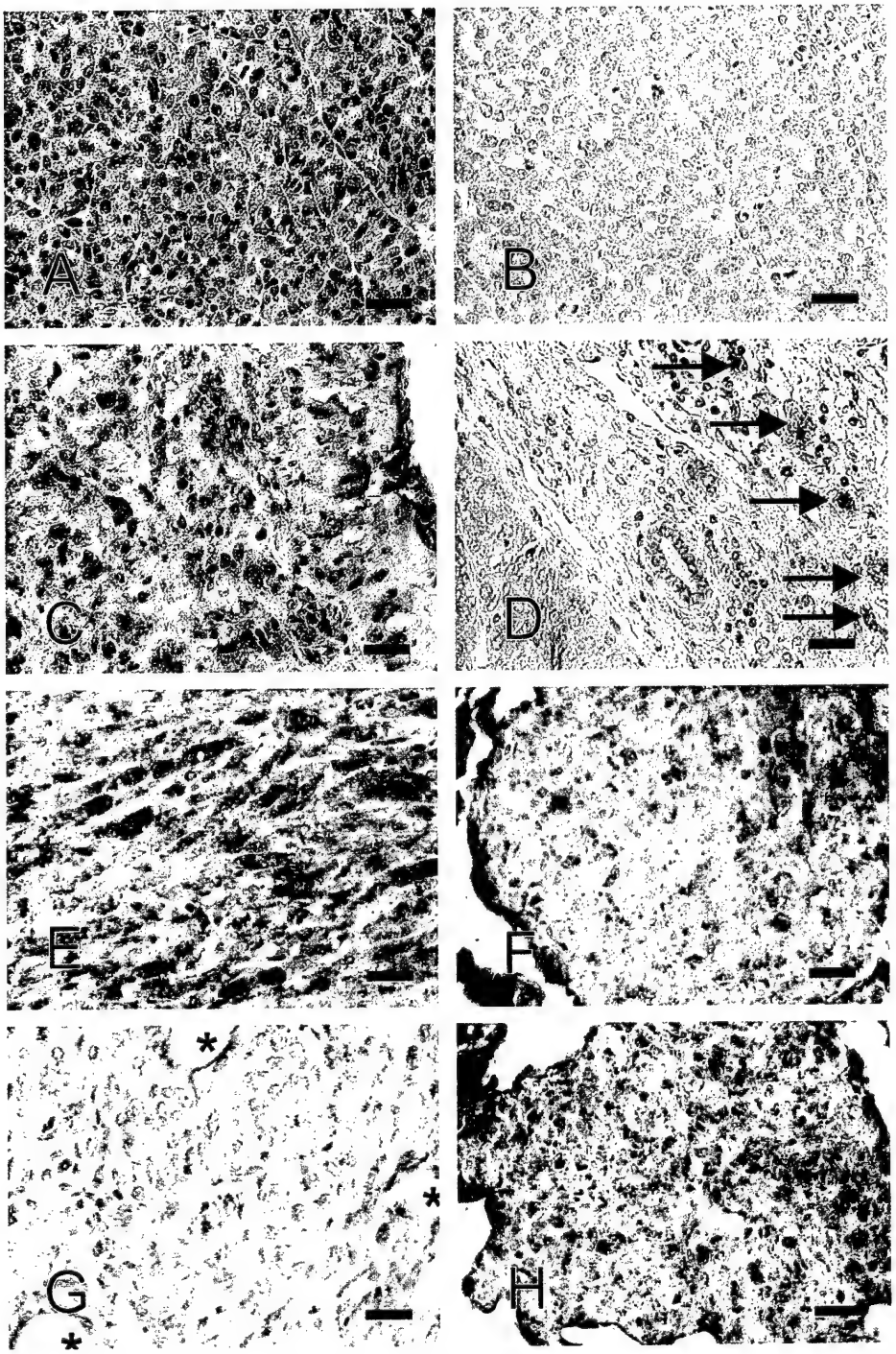


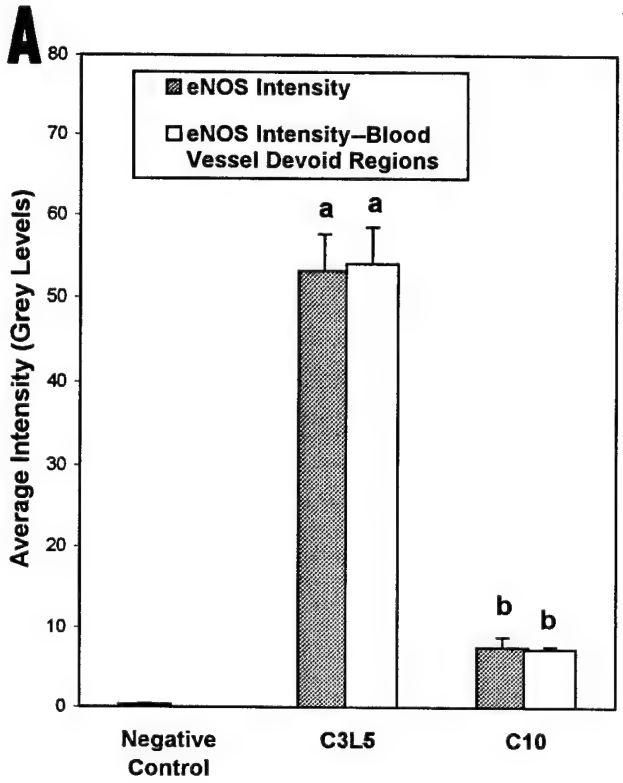
FIGURE 2 – Immuno-histochemical localisation of eNOS and iNOS proteins in spontaneous *(a–d)* and transplanted *(e–g)* mammary tumours (C3L5 or C10) at primary and metastatic sites. *(a)* Spontaneous primary tumour showing both eNOS-positive and -negative tumour cells arranged in pseudo-acinar formation. *(b)* Representative negative control. *(c)* Lung metastasis of tumour depicted in *(a)*; metastases were always strongly and homogeneously eNOS-positive. *(d)* Spontaneous primary tumour immunostained for iNOS; macrophages, present in tumour stroma, stained positively for iNOS, whereas tumour cells did not express iNOS. *(e)* C3L5 primary tumours transplanted s.c. exhibited strong and homogeneous eNOS positivity, whereas C10-derived tumours *(g)* showed weak and heterogeneous eNOS staining. Constitutive eNOS protein was expressed in endothelial cells lining blood vessels (* in *g*) in tumour tissue. eNOS expression was similar in metastatic lung colonies arising after C3L5 or C10 transplantation (*f* and *h*, respectively). Scale bars = 30 μm.

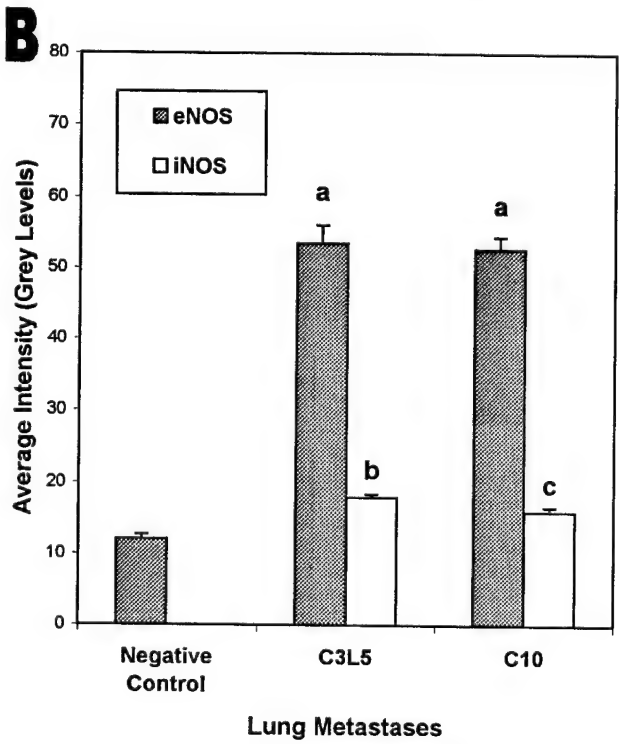
means for a single main effect were tested using 1-way ANOVA. Data from the *in vivo* pulmonary metastasis assay were analyzed using the Mann-Whitney rank sum test. Main effects of tumour type (*i.e.*, C3L5 and C10) and treatment (*i.e.*, L-NAME and D-NAME) were tested for the dependent variable (blood vessel formation) using 2-way ANOVA in the *in vivo* angiogenesis assay. A probability of 0.05 was always used in determining statistical significance.

RESULTS

Primary tumour growth rate and metastatic lung colony formation

Figure 1 shows the volume of primary tumours and the number of lung metastases forming in C3H/HeJ mice receiving s.c. implants of C3L5 or C10 tumour cells (n = 15 mice/group). C3L5-derived primary tumours grew faster, resulting in larger tumours, than those derived from C10 cells at day 14 ($p < 0.0001$) and day 21 ($p < 0.0001$) after tumour transplantation (Fig. 1*a*). In addition, the number of spontaneous metastatic lung colonies harvested at 21 days (Fig. 1*b*) was higher in mice receiving s.c. implants of C3L5 cells relative to those receiving C10 cells (C3L5 199.6 ± 25.0, C10 54.1 ± 18.3; $p < 0.001$).





Immuno-cytochemical detection of NOS (eNOS and iNOS) enzymes

Strong and homogeneous eNOS staining was observed *in vitro* in 100% of cultured C3L5 mammary adenocarcinoma cells, as reported by Orucevic *et al.* (1999), suggesting that these cells constitutively express high levels of eNOS. In contrast, *in vitro* eNOS immunostaining in C10 cells was much weaker and more heterogeneous relative to C3L5 cells (data not shown). Under normal culture conditions, C3L5 and C10 cells did not express iNOS; however, iNOS expression was induced in both cell lines using IFN-γ and LPS in approximately 40% to 50% of cells (data not shown), as previously reported for C3L5 cells (Orucevic *et al.*, 1999).

Spontaneous mammary tumours developing in C3H/HeJ female retired breeder mice (tumour diameter at 8 to 12 weeks' = 8 to 20 mm) showed localised variations in levels of morphological differentiation within primary tumours, irrespective of tumour growth rate. Differentiated zones were comprised of tumour cells arranged in pseudo-acini, whereas poorly differentiated sites constituted spindle-shaped tumour cells arranged in sheets, whorls or clusters. A heterogeneous pattern of eNOS expression was seen in both differentiated and poorly differentiated zones of tumour tissue; cells were either strongly eNOS-positive or completely eNOS-negative (differentiated zone, Fig. 2*a*); however, the proportion of eNOS-positive cells was consistently higher in poorly differentiated zones relative to differentiated zones (data not shown). Overall, approximately 40% to 70% of tumour cells in individual primary tumours were eNOS-positive. A clear correlation between eNOS expression patterns and tumour growth rates was not observed (high tumour growth rate = 13 to 20 mm tumour diameter at 8 to 12 weeks, n = 4; moderate growth rate = 8 to 12 mm, n = 11; low growth rate = <8 mm, n = 5). In contrast, all spontaneous lung metastases were strongly and homogeneously eNOS-positive; virtually all (76% to 100%) tumour cells at metastatic sites expressed eNOS (Fig. 2*c*). Tumour cells within primary and metastatic sites did not express iNOS; however, some tumour-associated macrophages, located in the primary tumour tissue and surrounding stroma (Fig. 2*d*) and occasionally at metastatic sites (data not shown), stained positively for iNOS.

Figure 2*e–h* shows eNOS staining in primary and metastatic tumours 3 weeks after transplantation of C3L5 (Fig. 2*e*, primary tumour; Fig. 2*f*, lung metastasis) and C10 (Fig. 2*g*, primary tumour; Fig. 2*h*, lung metastasis) cells. Primary tumours derived

FIGURE 3 – Quantification of the average staining intensity of primary tumours from C3H/HeJ mice bearing C3L5 or C10 tumour transplants immunostained for eNOS protein. *(a)* The intensity of immuno-histochemical staining for eNOS was higher in tumour cells within primary C3L5 tumours than in those derived from C10 cells ($p < 0.001$). eNOS immunostaining intensity did not differ in regions containing blood vessels *vs.* those devoid of blood vessels, and this was observed for both C3L5 and C10-derived tumours (C3L5 $p = 0.8969$, C10 $p = 0.9012$), indicating that endothelial cells did not alter the relative amount of eNOS staining between C3L5- and C10-derived tumours. *(b)* The intensity of eNOS immunostaining did not differ in lung metastases derived from C3L5 or C10 cells ($p = 0.8008$). iNOS immunostaining was slightly higher in lung metastases derived from C3L5 cells relative to those derived from C10 cells ($p < 0.0197$). Bars not sharing a common letter (*i.e.*, a, b or c) in each diagram are significantly different.

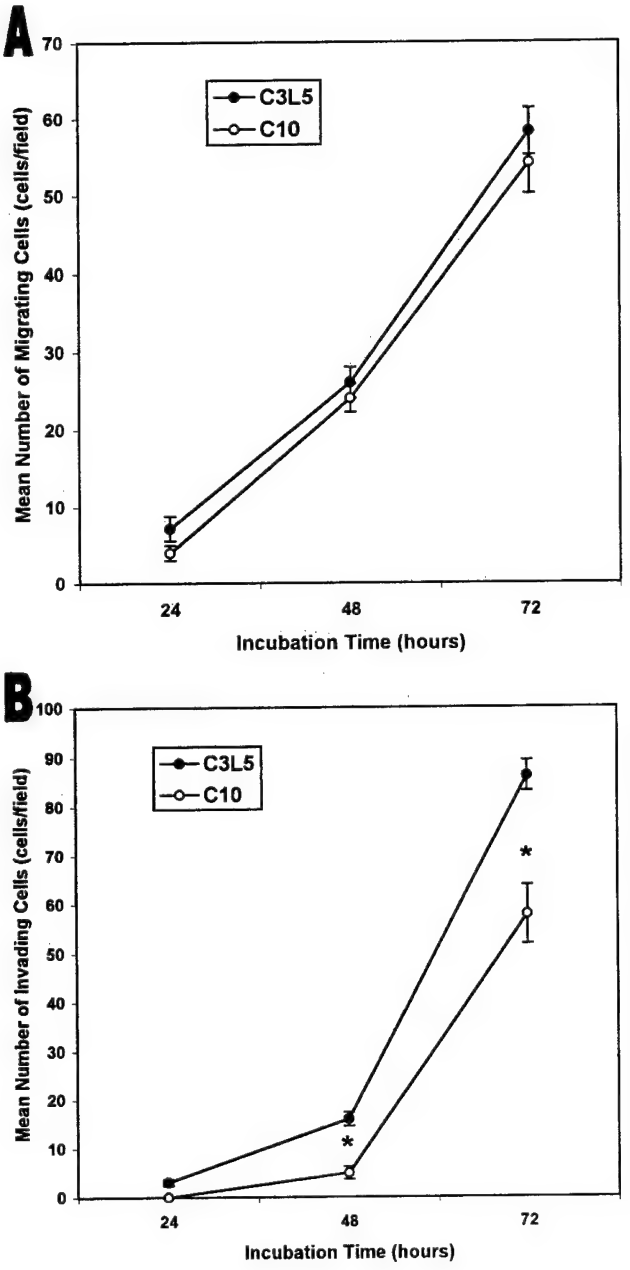


FIGURE 4 – Temporal kinetics of *in vitro* migration and invasion. *(a)* Kinetics of migration of C3L5 and C10 tumour cells; C3L5 and C10 cells did not differ in migratory capacity (n = 20 fields/group). *(b)* Kinetics of invasion of C3L5 and C10 tumour cells; invasion rate of C10 cells was slower than that of C3L5 cells (*$p < 0.0011$, n = 20 fields/group).

from the weakly metastatic C10 cell line showed weak and heterogeneous eNOS staining; relatively weak eNOS positivity was noted in approximately 20% to 50% of tumour cells (Fig. 2*g*). Primary tumours derived from the highly metastatic C3L5 cell line (Fig. 2*e*) were strongly and homogeneously eNOS-positive; most (>90%) tumour cells expressed eNOS. However, regardless of the tumour cell line transplanted (*i.e.*, C3L5 or C10), lung metastases were always strongly and homogeneously eNOS-positive; approximately 60% to 80% of tumour cells within metastatic lung colonies showed strong staining for eNOS (Fig. 2*f*, C3L5; Fig. 2*h*, C10). Cells within primary tumours and metastases did not express iNOS. However, iNOS expression was observed in tumour-associated macrophages at primary and metastatic sites of both tumour types (data not shown).

Quantification of NOS staining intensity of primary tumours and lung metastases

Figure 3 shows quantification of the staining intensity within primary, healthy (non-necrotic) tumour tissue and lung metastases (Fig. 3*a,b*) harvested 3 weeks after transplantation of C3L5 or C10 cells. The average intensity of immuno-histochemical staining for eNOS was higher in cells within primary tumours derived from C3L5 cells relative to those derived from C10 cells (C3L5 53.2 ± 4.9, C10 7.5 ± 1.3; $p < 0.001$). Since eNOS staining intensity did not differ in regions of tumour sections containing blood vessels compared with those devoid of blood vessels and this relationship was observed for both C3L5- and C10-derived tumours (C3L5 blood vessel–containing regions = 53.2 ± 4.9, blood vessel–devoid regions = 54.0 ± 4.5, $p = 0.8969$; C10 blood vessel–containing regions = 7.5 ± 1.3, blood vessel–devoid regions = 7.3 ± 1.2, $p = 0.9012$), it appears that endothelial cells did not alter the relative amount of eNOS staining between C3L5- and C10-derived tumours. The intensity of eNOS immunostaining did not differ in lung metastases derived from C3L5 and C10 cells (C3L5 53.5 ± 2.6, C10 52.6 ± 1.8; $p = 0.8008$). iNOS immunostaining was higher in metastatic lung colonies derived from C3L5 relative to those derived from C10 cells (C3L5 17.9 ± 0.4, C10 15.9 ± 0.7; $p > 0.0197$).

Kinetics of in vitro *migration and invasion by C3L5 and C10 cells*

The temporal kinetics of migration and invasion are shown in Figure 4. Migration rates of the 2 cell types did not differ ($p = 0.429$ at 72 hr); however, C3L5 cells were more invasive than C10 cells (C3L5 86.2 ± 3.6, C10 57.8 ± 6.9; $p < 0.0011$ at 72 hr).

Migration and invasiveness under different treatment conditions

The effects of L-NAME treatment at various doses ± 5-fold excess L-arginine (natural substrate for NOS, competes with and blocks NO-specific effects of L-NAME) on the migratory and invasive abilities of both cell lines at 24, 48 and 72 hr were examined. Since the effects were qualitatively similar at all time points, only the 72 hr time point is shown (Fig. 5). L-NAME treatment at varying doses (0.01, 0.1 and 1 mM) reduced the migratory capacity of both C3L5 ($p < 0.0001$) and C10 ($p < 0.0001$) cells relative to untreated control cells. Migratory capacities of both cell lines were restored to baseline levels after additional treatment with excess L-arginine, indicating that the inhibitory effects of L-NAME on migration were NO-specific (Fig. 5*a*).

The invasion indices of C3L5 and C10 cells after 72 hr incubation, under different treatment conditions, are shown in Figure 5*b*. As shown earlier, C3L5 cells invaded the Matrigel barrier at a faster rate than C10 cells ($p < 0.0011$). In addition, L-NAME treatment at the various doses reduced the invasive capacity of both C3L5 ($p < 0.0001$) and C10 ($p < 0.0001$) cells relative to untreated control cells. Invasive capacities of both cell lines were restored to near baseline levels after additional treatment with excess L-arginine, indicating that the effects of L-NAME on invasion were NO-specific.

NO production assay

Figure 6 shows the levels of NO produced by C3L5 and C10 cells as measured by the nitrate/nitrite levels present in the culture media collected after 72 hr incubation under the treatment conditions examined in invasion and migration assays. Under normal culture conditions, nitrate/nitrite levels were higher in culture media from C3L5 cells compared with C10 cells (C3L5 78.4 ± 1.2, C10 67.4 ± 0.5; $p < 0.0001$). For both cell lines, treatment of cells with L-NAME at the various doses reduced levels of nitrate/nitrite produced relative to untreated control cells (C3L5 $p <$

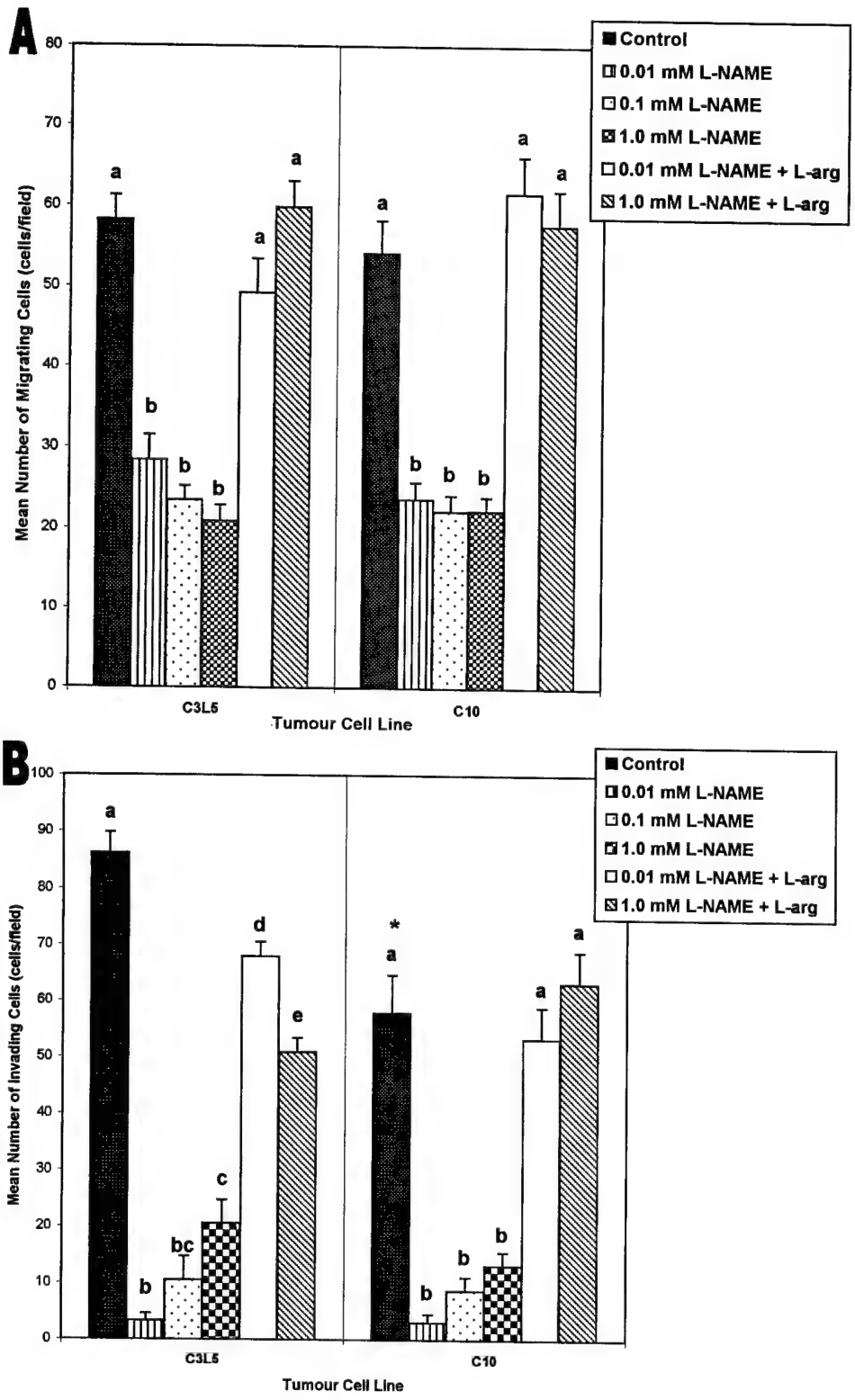


FIGURE 5 – Migration and invasion indices of C3L5 and C10 cells under different treatment conditions (72 hr incubation). *(a)* Migratory ability of both C3L5 and C10 cells was reduced after treatment with L-NAME at various concentrations (0.01, 0.1 and 1 mM) relative to control cells (C3L5 $p < 0.0001$, C10 $p < 0.0001$), and migration of both cell lines was restored to basal levels after additional treatment with L-arginine (0.01, 1.0 mM). *(b)* Basic invasiveness of C10 cells was lower (*) than that of C3L5 cells. Treatment of C3L5 and C10 cells with L-NAME reduced invasive ability relative to untreated controls (C3L5 $p < 0.0001$, C10 $p < 0.0001$). Additional treatment with L-arginine increased invasion of both cell lines, restoring it to near basal levels. Within each cell line, bars not sharing a common letter (*i.e.*, a–e) are significantly different.

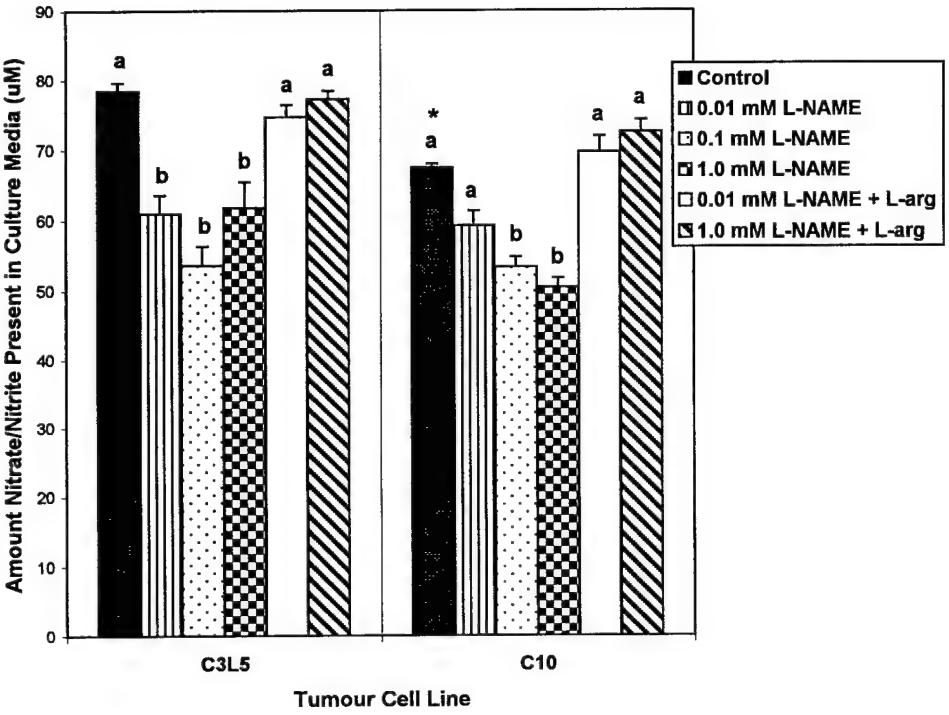


FIGURE 6 – NO production by C3L5 and C10 cells measured by nitrate/nitrite levels in culture media under different treatment conditions (72 hr incubation). Concentrations of nitrate/nitrite were higher for C3L5 than for C10 cells ($*p < 0.0001$). Relative to untreated C3L5 and C10 cells, production of NO by both cell lines decreased significantly after treatment with L-NAME at various concentrations (0.01, 0.1, 1.0 mM; C3L5 $p < 0.0001$, C10 $p < 0.0001$). Levels of nitrate/nitrite increased and were restored to near basal values with additional exposure of C3L5 and C10 cells to L-arginine (0.01, 1.0 mM). Bars not sharing a common letter (*i.e.*, a or b) are significantly different.

0.0001, C10 $p < 0.0001$), and these levels were restored with additional exposure of cells to excess L-arginine.

In vivo *tumour-induced angiogenesis assay*

Figure 7 shows the number of blood vessels per unit area in Matrigel implants containing C3L5 or C10 cells, retrieved from animals treated with L-NAME or D-NAME (control animals). C3L5 cells were more angiogenic than C10 cells; in control animals treated with D-NAME, neovascularisation was lower in C10-containing implants than in those containing C3L5 cells (C3L5 71.9 ± 5.33, C10 39.1 ± 4.9; $p < 0.0001$). L-NAME therapy reduced angiogenesis in C3L5-containing implants relative to control animals but did not affect neovascularisation in C10-containing implants (C3L5 L-NAME 34.2 ± 4.7, D-NAME 71.9 ± 5.3, $p < 0.0001$; C10 L-NAME 38.1 ± 4.7, D-NAME 39.1 ± 4.9, $p = 0.8903$).

DISCUSSION

We have investigated the role of endogenous NO, resulting from eNOS expression by tumour cells, in tumour progression and metastasis using a C3H/HeJ murine mammary adenocarcinoma model that includes spontaneously arising tumours and 2 clonal derivatives that differ in metastatic phenotype. Examination of a large number of spontaneous tumours confirmed our preliminary findings (Lala and Orucevic, 1998) that tumour cells at primary sites were distinctly heterogeneous in eNOS protein expression, whereas those at spontaneous lung metastatic sites had a strong and homogeneous expression pattern, suggestive of a metastasis-promoting role of eNOS. This concept was further validated by our findings of a positive correlation between levels of eNOS expression in primary tumours and primary tumour growth rate and formation of spontaneous pulmonary metastases, using 2 transplanted, clonally derived cell lines: C10, a weakly metastatic cell line (Lala *et al.*, 1986), and C3L5, a highly metastatic cell line (Lala and Parhar, 1993).

The 2 cell lines differed in eNOS expression *in vitro* and *in vivo* at primary transplant sites. However, as revealed using objective, computer-assisted quantification, eNOS expression in tumour cells did not differ at metastatic sites. Exclusion of eNOS-positive vascular endothelial cells from the analysis did not alter *in vivo* results, suggesting that tumour-derived eNOS contributed to the observed variations in eNOS expression between the 2 cell lines. Furthermore, because iNOS expression (observed in macrophages within primary tumour tissue, in surrounding stroma and at metastatic sites) did not differ between C3L5 and C10-derived tumours, it is likely that the observed differences in tumour growth and metastasis were eNOS-mediated. However, this relationship, on its own, did not establish causality. A causal relationship between NO production and tumour progression was earlier demonstrated in C3L5 tumour-bearing mice; anti-tumour and anti-metastatic effects were observed with NOS inhibition using NMMA and L-NAME (Orucevic and Lala, 1996*a,b*). The present study further explored the underlying mechanisms utilising both tumour cell lines.

Key cellular processes that determine primary tumour growth rate are tumour cell proliferation, survival and capacity for angiogenesis; invasiveness and metastasis depend on migration and matrix degradation by tumour cells. We earlier found that endogenous NO did not alter C3L5 cell proliferation (Orucevic *et al.*, 1999); we have also observed that *in vitro* growth rates of C10 and C3L5 cells did not differ (data not shown). In the present study, we compared migratory, invasive and angiogenic capacities of the 2 cell lines and the role of endogenous NO in these processes.

Despite differences in eNOS expression and NO production by C3L5 and C10 cell lines *in vitro,* the migratory capacities did not differ. However, for both cell lines, migration and NO production

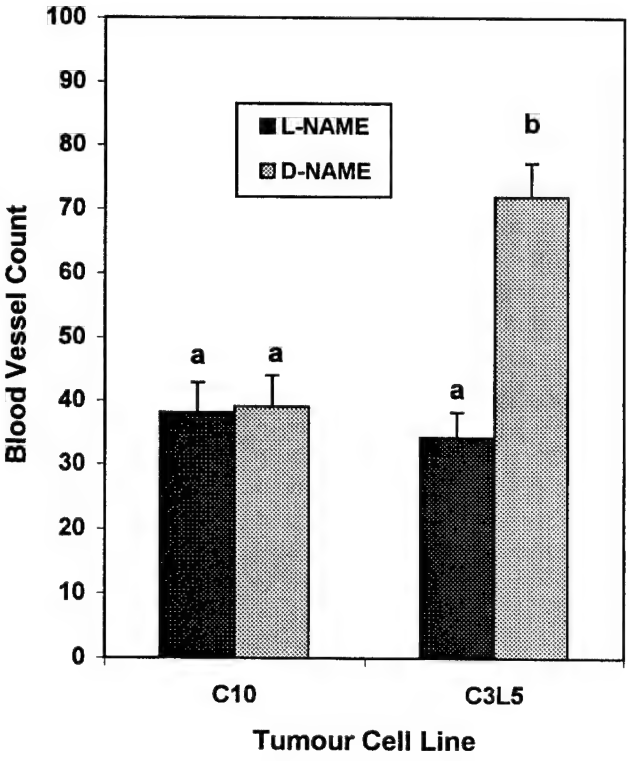


FIGURE 7 – Levels of angiogenesis in C10 and C3L5 implants in L-NAME–treated and control (D-NAME–treated) animals. For control animals treated with D-NAME, the neovascular response was higher in Matrigel implants containing C3L5 cells than in those containing C10 cells ($p < 0.0001$). L-NAME treatment reduced angiogenesis in implants containing C3L5 cells relative to control animals ($p < 0.0001$) but did not alter angiogenesis in implants containing C10 cells relative to control animals ($p = 0.8903$). Bars not sharing a common letter (*i.e.*, a or b) are significantly different.

were inhibited in the presence of L-NAME and inhibitory effects were abrogated with additional exposure of cells to L-arginine, indicating that endogenous NO stimulated migration. Thus, the absence of differences in the basal migration rates may be explained by the presence of additional migration regulatory factor(s) differentially expressed by the 2 cell lines. The present report demonstrates a migration-promoting role of endogenous, tumour-derived NO. The precise pathway of signal transduction responsible for this role remains to be examined.

C3L5 cells were more invasive than C10 cells, consistent with the differences in their metastatic capacities *in vivo*. For both cell lines, invasiveness and NO production were suppressed with L-NAME and restored with additional exposure of cells to L-arginine, suggesting that endogenously-derived NO promoted invasion. Therefore, varied capacities for invasion could be explained, in part, by differences in NO production between the 2 cell lines. Because cellular invasiveness depends on multiple steps, including matrix degradation and migration, and migration did not differ between the 2 cell lines, it is likely that the differences in their invasiveness were due to differences in their matrix-degrading capabilities. Orucevic *et al.* (1999) demonstrated that endogenous NO promoted matrix degradation by C3L5 cells by altering the balance between matrix metalloproteases (MMPs) and their natural inhibitors, tissue inhibitors of metalloproteases (TIMPs): constitutively produced NO by C3L5 cells down-regulated TIMP-2 and TIMP-3 mRNA, whereas induction of additional NO production by LPS and IFN-γ up-regulated MMP-2 mRNA. Additional mechanisms of NO-mediated stimulation of cellular invasiveness may exist. For example, NO was shown to stimulate degradation of articular cartilage by stimulating other MMPs (collagenases and stromelysin) in human, bovine and rabbit chondrocytes (Murrell *et al.*, 1995; Tamura *et al.*, 1996).

Finally, C3L5 and C10 cells differed in their angiogenic capacities *in vivo*, consistent with differences in their NO-producing abilities. This may partly explain the observed differences in primary tumour growth rates following transplantation of equivalent numbers of C3L5 or C10 cells at identical sites in syngeneic mice. For C3L5 cells, endogenous NO promoted vascularisation; angiogenesis was markedly reduced in L-NAME–treated mice relative to those receiving D-NAME, confirming earlier findings using the novel *in vivo* angiogenesis assay devised in our laboratory (Jadeski and Lala, 1999). However, in C10-derived Matrigel implants, L-NAME treatment did not reduce the angiogenic response relative to D-NAME treatment. Two explanations may be offered for this finding: *(i)* the cell lines differentially express other angiogenesis-regulating factor(s) and *(ii)* a certain threshold level of NO is required to stimulate angiogenesis in the present tumour model. Angiogenesis promotion by NO has been reported utilizing *in vitro* and *in vivo* assays (Ziche *et al.*, 1994, 1997) and has been demonstrated in numerous tumour models. For example, iNOS over-expression in a human colonic adenocarcinoma cell line led to increased growth rate and enhanced vascularity of transplanted tumours in nude mice (Jenkins *et al.*, 1995). Furthermore, NOS inhibition reduced angiogenesis in the rabbit cornea following xenotransplantation of human squamous-cell carcinoma cells (Gallo *et al.*, 1998). Taken together, these findings suggest that promotion of angiogenesis is a key event responsible for NO-mediated stimulation of tumour growth and metastasis.

In summary, our results in the C3H/HeJ murine mammary tumour model have established that tumour-derived NO plays a stimulatory role in tumour progression and metastasis by multiple mechanisms: promotion of migration, matrix degradation and angiogenesis. Thus, NOS inhibitors may prove to be important components of combination therapy protocols in certain human tumours, including breast cancer, which exhibits a positive association of NOS activity with tumour grade (Thomsen *et al.*, 1995; Dueñas-Gonzalez *et al.*, 1997).

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Role of nitric oxide in carcinogenesis and tumour progression

Peeyush K Lala and Chandan Chakraborty

Nitric oxide (NO) is a short-lived molecule required for many physiological functions, produced from L-arginine by NO synthases (NOS). It is a free radical, producing many reactive intermediates that account for its bioactivity. Sustained induction of the inducible form of NOS (iNOS) in chronic inflammation may be mutagenic, through NO-mediated DNA damage or hindrance to DNA repair, and thus potentially carcinogenic. Expression of iNOS is positively associated with *P53* mutation in tumours of the colon, lung, and oropharynx. Progression of a large majority of human and experimental tumours seems to be stimulated by NO resulting from activation of iNOS or constitutive NOS, whereas inhibition is documented in others. This discrepancy is largely explained by differential sensitivity of tumour cells to NO-mediated cytostasis or apoptosis and clonal evolution of NO-resistant and NO-dependent cells. *P53* mutation or loss is one of many events linked with NO resistance and dependence. NO can stimulate tumour growth and metastasis by promoting migratory, invasive, and angiogenic abilities of tumour cells, which may also be triggered by activation of cyclo-oxygenase (COX)-2. Thus, selective inhibitors of NOS, COX, or both may have a therapeutic role in certain cancers.

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The discovery in 1987 that nitric oxide (NO) accounted for the bioactivity of endothelium-derived relaxing factor (EDRF)[1,2] rapidly led to an explosion of information on the physiological and pathological roles of this molecule. Although most well known for its physiological roles in vasorelaxation, neurotransmission, inhibition of platelet aggregation, and immune defence, NO also acts as an intracellular messenger for various cells in almost every system in the body. Many reviews are available on different features of NO, including a few in tumour biology.[3,4] This review attempts to integrate the apparently conflicting information on the roles of NO in carcinogenesis and tumour progression. Cellular mechanisms in tumour progression are summarised in Figure 1.

NO is a short-lived (half-life 3–30 s) colourless gas that is moderately soluble in water (up to 2 mmol/L) but highly soluble in organic solvents.[1,2] Its lipophilic nature means that it can diffuse between cells very easily. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS). The three main isoforms are neuronal (n) NOS, inducible (i) NOS, and endothelial (e) NOS. Generally, nNOS and eNOS

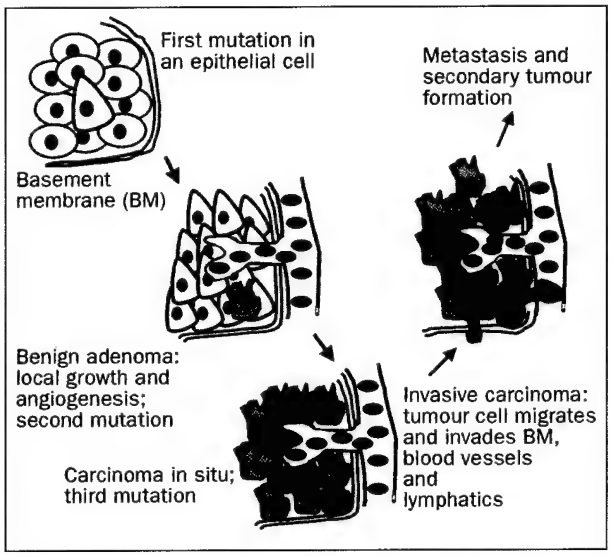

Figure 1. Typical chronology of cellular events during tumour progression.

are expressed constitutively in neurons and endothelial cells, respectively, though they can also be expressed by other cells. Activation of these two isoforms depends on calcium ions and calmodulin, resulting in NO production in low concentrations (usually nanomolar). Expression of iNOS, by contrast, typically requires induction by bacterial products alone or with inflammation-associated cytokines in many cell types, particularly macrophages. Activation of iNOS does not require calcium ions and calmodulin; activation of this enzyme leads to production of high concentrations of NO (generally micromolar), which may be sustained for a long period. eNOS knock-out mice show systemic hypertension, consistent with the role of endothelial NO in reducing vascular tone. iNOS knock-out mice are prone to infection, and their macrophages have poor cytotoxicity against parasites and tumour cells, consistent with recognised roles of NO derived from neutrophils and macrophages in killing bacteria, parasites, and certain tumour cells. In nNOS knock-out mice, both sexes can have hypertrophic pyloric stenosis and males show aberrant mounting behaviour, consistent

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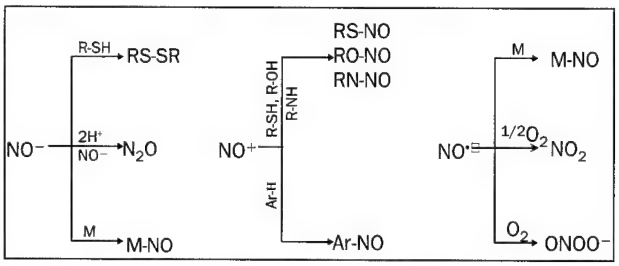

Figure 2. Formation of different reactive products from nitric oxide. Pleiotropic biological effects of NO may depend upon this reactivity.

with the role of neuron-derived NO in relaxing pyloric sphincter muscles and neurotransmission.

NO has an unpaired electron, hence is a free radical (NO). NO becomes nitrosonium cation (NO+) or nitroxyl anion (NO-) by donating or accepting an electron, respectively.[5] In a living system, NO- can undergo spontaneous dimerisation to form dinitrogen oxide (N_2O), or it can react with thiols, resulting in oxidation of sulphydryl, or with metals (Figure 2). NO+ is involved in nitrosation reactions with nucleophilic groups such as thiols, amides, carboxyls, hydroxyls, and aromatic (Ar) rings. NO· can react with O_2 to form nitrogen dioxide (NO_2-), with O_2- to form peroxynitrite (ONOO-), or with transition metal ions to form metal-nitrosyl (M-NO) complexes. Most of these products are more reactive than NO·. For example, ONOO-, although less stable and shorter-lived than NO·, is much more cytotoxic – for instance, in causing DNA damage. Thus, chemoprotection against NO-mediated cellular injury might be achievable with NOS inhibitors, antioxidants, and peroxynitrite scavengers.

Role of NO in carcinogenesis

Transition of a normal somatic cell to a cancer cell is generally the result of many genetic changes, involving activation of oncogenes or inactivation of tumour-suppressor genes. These changes allow the cell to escape normal control mechanisms in cell proliferation, differentiation, migration, and death, which collectively maintain the normal cellular architecture and functions in an organised tissue. The frequency of somatic mutations leading to carcinogenesis in human beings is dictated largely by chemicals in the cellular microenvironment and to a small extent by heritable genetic predisposition.

Because NO has mutagenic properties, long-term exposure of cells to high NO concentrations resulting from iNOS induction during chronic inflammation could have an active role in carcinogenesis.[3,6] This contention is supported by a model of inflammation in transgenic mice carrying the *Lac-Z* gene, in which B-cell lymphoma transplants localising to the spleen caused an increase in mutation frequency of this gene in the splenic cells, which was alleviated with NOS inhibitors.[7] Mutagenesis by NO can occur through several mechanisms. First, DNA damage due to deamination of nucleic acid bases has been shown in cell-free systems, bacteria,[8] and macrophages.[9] Second, transition and or transversion of nucleic acid bases (eg G:C→T:A; G:C→C:G; G:C→A:T) by reactive NO products has been documented in plasmid DNA.[10] Third, inactivation of DNA-repair proteins (eg alkyltransferase[11] and DNA ligase[12]) can occur owing to the high affinity of reactive NO species for aminoacids containing thiol residues. Although a carcinogenic role of NO and reactive NO species has been inferred primarily by extrapolation from their mutagenic role, direct evidence for involvement of NO in chemically induced transformation of C3H10Tfi mouse fibroblast cells has been reported.[13] Other evidence suggests that NO may promote carcinogenesis by inactivating the tumour-suppressor oncoprotein P53. Cells containing wild-type *P53*, when exposed to excess NO, accumulated P53 protein,[14] with a concomitant loss of its DNA-binding activity,[15] which has been attributed to nitration of tyrosine residues in the protein.[16] Further mutation of *P53* may occur in a high NO environment. Indeed, a positive correlation between total NOS activity and frequency of *P53* mutation was reported in many cases of lung adenocarcinoma. A strong association between iNOS activity and the prevalent form of *P53* mutation (G:C→A:T at CpG dinucleotides) was reported in a study of 118 sporadic human colon tumours[17] and 27 carcinomas of the head and neck.[18] Although a cause and effect relation between these two features has been debated,[18] peak iNOS activity noted in colonic adenomas before transition into carcinomas supports a carcinogenic role.[17] Increased NOS activity underlying metaplastic changes has been suggested for breast apocrine metaplasia of fibrocystic disease[19] and hyperplasia of stromal tissue in Barrett's oesophagus.[20]

Role of NO in tumour progression

Progression of a newly developed tumour involves sequential changes in both cellular phenotype and genotype (Figure 1). These changes have been best correlated during the progression of colonic tumours. Phenotypically, these tumours appear as non-invasive benign adenomatous polyps, some of which progress into invasive carcinomas, and metastatic spread of carcinoma cells to distant organs follows. Some of the genetic changes associated with these phenotypically defined stages have been identified. Tumour progression is the end result of collective behaviour of cells involving multiple cellular processes: cell proliferation and survival, migration and invasiveness, and cellular ability to induce angiogenesis. Molecular events underlying each of these functions represent areas of extensive research.

A solid tumour consists of tumour cells and host-derived cells, including tumour-infiltrating leucocytes and cells of the tumour vasculature, especially endothelial cells. One or more of these cellular constituents may express any of the active NOS isoforms, serving as a source of NO in the tumour microenvironment. Functional roles of tumour-derived NO in tumour progression represent a composite of NO-mediated effects on: tumour-cell proliferation, survival, migration, and invasiveness; the function of immune cells infiltrating tumours; and the endothelial-cell progenitors able to induce angiogenesis. A dissection of these roles may explain much of the conflict in published findings of a positive as well as a negative association of NO with tumour progression, documented in both human and experimental models.

Positive association of NO with tumour progression

This type of association has been overwhelming for many human and experimental tumours. The amount of immunoreactive NOS protein, its activity, or both, in the tumour has been positively related to the degree of malignancy for tumours of the human reproductive tract,[21] breast[22,23] and central nervous system.[24] In breast cancer, iNOS expression by macrophages, stromal cells, and tumour cells[22,23,25] accounted for most of the NOS activity. In a sample of 111 tumours (43 *in situ* and 68 invasive breast carcinoma), there was a strong association between iNOS positivity of stromal cells and/or tumour cells and local microvascular density and apoptotic indices, both of which are indicators of poor prognosis.[25] In apparent contrast, researchers who reported a retrospective study of 118 patients with breast cancer proposed that expression of eNOS protein in tumour cells and peritumoral microvessels may be a favourable prognostic indicator in premonopausal women.[26] However, lack of information on iNOS expression and total NOS activity in that study precludes association between tumour-derived NO and tumour progression. In most gastric carcinomas, iNOS was detected in stromal elements and eNOS in the tumour vasculature.[3] iNOS expression was higher in prostatic carcinomas than in benign prostatic hyperplasia.[27] Similarly, compared with healthy control tissue, total NOS activity was higher in lung adenocarcinomas[28] and carcinomas of the larynx, oropharynx, and oral cavity.[29] In the last study,[29] NOS expression was positively associated with tumour microvessel density and angiogenic ability of tumours in rabbit corneal xenografts.

Experimental tumour models have provided more convincing evidence for a direct role of NO in tumour growth and metastasis. In a rat adenocarcinoma model, in which endothelial cells of the tumour vasculature expressed iNOS, treatment of hosts with NG-nitro-L-arginine methyl ester (L-NAME) decreased NO production and tumour growth. Furthermore, despite *in vitro* cytostatic effects of NO induced with lipopolysaccharide and interferon γ in EMT-6 murine mammary tumour cells, this induction promoted growth and metastasis of tumour cells *in vivo*.[30] Similarly, iNOS transduction in a human colonic adenocarcinoma line led to stimulation of tumour growth and vascularity in nude mice,[31] which was abrogated by treatment with a selective iNOS inhibitor, 1400W.[32] Finally, NO-mediated stimulation of tumour growth and metastasis was documented in a murine mammary adenocarcinoma model, which included C3H/HeJ spontaneous mammary tumours and their clonal derivatives showing high and low metastatic abilities.[3] The spontaneously developing tumours showed a variable mixture of eNOS-positive and eNOS-negative tumour cells, whereas the metastatic cells were predominantly eNOS-positive. These findings suggest that eNOS expression promoted metastatic behaviour. This hypothesis was validated by experiments designed with two cell lines clonally derived from a spontaneous tumour, one highly metastatic (C3L5) and one weakly so (C10).[33] They showed parallel differences in eNOS expression *in vitro* and only at the primary sites of transplantation *in vivo*, but their metastatic counterparts were strongly and equally eNOS positive. These tumour lines also showed parallel differences in growth rates at primary sites of transplantation, invasive behaviour *in vitro*, and angiogenic abilities *in vivo*.[33]

A cause and effect relation between NO production and tumour growth and metastasis was established in the above tumour model from the findings that both primary tumour growth and metastasis of the high-eNOS-expressing, highly metastatic C3L5 tumour line were decreased by treatment of mice with NOS inhibitors NG-methyl-L-arginine (NMMA) or L-NAME.[3] The mechanisms underlying NO-mediated tumour progression were identified subsequently in this tumour model as: promotion of tumour-cell invasiveness, resulting from differential regulation of matrix metalloproteases and their inhibitors;[34] promotion of tumour-cell migratory ability;[33] and promotion of tumour-induced angiogenesis.[35] Despite differences in eNOS expression and NO-producing abilities between C3L5 and C10 cells, L-NAME treatment inhibited invasive and migratory functions of both cell lines *in vitro*; these functions were restored in the additional presence of excess L-arginine, which implicates NO-mediated effects.[33] Angiogenic ability *in vivo* was decreased by L-NAME therapy in high-eNOS-expressing C3L5 cells[35] but not in low-eNOS-expressing C10 cells.[33]

Inverse association between NO and tumour progression

The amount of NOS protein and NOS enzyme activity were reported to decline during the transition of human colonic mucosa to polyps and then to carcinomas.[36] However, as discussed earlier, similar findings by Ambs and colleagues[37] and their correlation with *P53* mutation[17] were interpreted as high NOS activity in colonic adenomas contributing to *P53* mutation as well as angiogenic responses promoting transition to carcinomas. In various murine melanoma cell lines, NOS activity was inversely correlated with metastatic ability.[3] When genetically transduced to overexpress iNOS, melanoma cells[3] and renal carcinoma cells[38] lost their tumorigenic and metastatic abilities as a result of NO-mediated tumour-cell apoptosis. Furthermore, tumour cells transduced with the *iNOS* gene can also destroy bystander tumour cells through NO-mediated antitumour cytotoxicity. Thus, this form of gene therapy may succeed even with a low transfection efficiency.[39]

Possible reasons for the conflicting roles of NO in tumour progression

Of the many variables that may explain the discrepancies in the results described above, the most important seems to be the genetic constitution of cells that may determine NO sensitivity or resistance. We have proposed that the genetic make-up of tumour cells and the concentrations of NO in the tumour-cell microenvironment are the main determinants of the role of NO.[3] During clonal evolution of tumours in the presence of high NO concentrations, NO-sensitive cells may be deleted and NO-resistant cells may emerge owing to mutational events mediated by NO.[3] *P53* mutation seems to be an important event leading not only to NO resistance but also to the ability of cells to use NO for

stimulating cellular processes that bring about tumour progression.[3] NO resistance may also be conferred by various protective mechanisms, such as activation of cyclo-oxygenase (COX)-2, the amount of functional Cu,Zn-superoxide dismutase, upregulation of heat-shock proteins, and concentrations of reduced glutathione. Irrespective of the mechanisms involved, NO resistance may also lead to NO dependence. This view is supported by the findings of Shi and colleagues,[40] who compared the growth of two murine tumour cell lines, M5076 ovarian sarcoma (NO sensitive) and B16-BL6 melanoma (NO resistant) in wild-type and *iNOS* null mice; absence of host iNOS promoted growth and metastasis of the former but retarded growth and metastasis of the latter cell line.

NO and tumour-cell proliferation/survival

There is no compelling evidence that NO can directly stimulate tumour-cell proliferation. By contrast, cytostatic and cytotoxic roles have been demonstrated. NO stimulates the accumulation of P53 protein in many cells, at least partly by inhibition of proteosomal degradation of the protein.[41] Because P53 causes transcriptional activation of many genes that lead to cell-cycle arrest (eg *P21*, cyclin G) and apoptosis (eg *BAX* and *FAS*), NO at high concentrations can cause P53-dependent cell-cycle arrest (cytostasis) as well as apoptosis.[4] Furthermore, NO can also cause cell death by necrosis as shown by an increase in lactate dehydrogenase activity.[42] The choice between apoptotic and necrotic pathways may be determined by the intracellular amount of non-haeme iron, which can protect from necrotic death.[43] NO-mediated cytostasis and apoptosis are recognised as part of the mechanisms leading to antitumour cytotoxicity of macrophages,[3] but not lymphokine-activated killer cells produced by interleukin-2 activation of lymphocytes.[44] NO-mediated killing of tumour cells has been reported for cytokine-activated endothelial cells, which suggests an antimetastatic mechanism for cytokine therapy.[45]

NO and tumour-cell apoptosis

The apoptotic process generally starts with the release of cytochrome C from mitochondria into the cytosol, where it binds to an adaptor molecule, apoptotic-protease-activating factor 1. The resultant complex activates the initiation caspases[8,9,10] which in turn, activate execution caspases,[3,6,7] and these catalyse the final events, DNA fragmentation and formation of apoptotic bodies.

The role of NO in tumour-cell apoptosis and survival depends on the cell type, the concentration of NO in the cellular microenvironment, the time of cellular exposure to NO, and possibly other factors. Of the possible genetic determinants that make a cell sensitive or resistant to NO, *P53* seems to be a major player. Generally, induction of apoptosis requires high concentrations of NO, typically produced in the presence of iNOS. By contrast, low concentrations of NO, typically produced in the presence of eNOS (or nNOS) can protect cells from apoptosis.

Functional P53 has a dual role in NO-mediated apoptosis: protection from apoptosis at low NO concentrations, and stimulation of apoptosis when NO concentrations are high. MCF7 breast-cancer cells expressing wild-type P53 and low eNOS activity resisted apoptosis induced by sodium butyrate, which was abrogated with NOS inhibitor or NO scavenger.[46] These cells also resisted apoptosis at low doses of exogenous NO, and this resistance was broken at high doses. The eNOS promoter region has a specific binding site for P53. In an independent study, the P53-mediated antiapoptotic role of NO in the same cells was shown to involve suppression of cytochrome c release from the mitochondria, inhibition of caspase 3, and an increase in BCL2 protein.[47] Indeed, low amounts of NO required for physiological functions may also serve to protect from apoptosis. In hepatocytes, this protection is mediated by S-nitrosylation of procaspases and active caspase enzymes.[48]

Many studies have shown upregulation of P53 protein before apoptosis induced by high NO concentrations resulting from exogenous NO or high iNOS expression. In turn, P53 can also downregulate iNOS by inhibiting the enzyme's promoter activity, thus decreasing NO-mediated genotoxicity.[4] Apoptosis is another protective device by which P53 may help eliminate cells with damaged DNA. In support of the requirement for functional P53 for high NO-induced apoptosis, Ho and colleagues[49] showed that cancer cell lines with wild-type *P53* (Hep G2, COLO 205) were vulnerable, whereas those with mutated (HT-29) or missing (Hep 3B) *P53* were resistant. About half of all human cancers show *P53* mutations, so these findings, if validated in other tumour lines, may serve as a valuable marker for NO sensitivity or resistance in human tumours. However, there is also evidence for P53-independent mechanisms of NO-induced apoptosis. For example, direct NO-mediated DNA fragmentation was documented in a human promyelocytic leukaemia cell line (U937) that lacks *P53*.[50]

Role of other events in NO resistance or sensitivity to apoptosis

COX-2 is involved in conferring NO resistance.[51,52] When NO-sensitive cells (RAW264.7 macrophage line) were transfected with a COX-2 expression vector[51] or pretreated with small amounts of NO to induce the enzyme through the activation of nuclear factor-κB and activator protein-1[52] the cells became resistant to high doses of NO.

The amount and activity of Cu,Zn-superoxide dismutase dictated the outcome of NO challenge to a human neuroblastoma cell line SH-SY5Y, transfected with a functional or mutant form of the enzyme.[53] Cells carrying the wild-type superoxide dismutase were protected, whereas those with mutant forms showed the typical events in the apoptotic cascade.

Upregulation of concentrations of reduced glutathione appeared to confer NO resistance to apoptosis in Jurkat lymphoma cells.

The ultimate role of NO in tumour progression or regression goes far beyond tumour-cell apoptosis, which is one of many events dictating tumour progression. Although the precise role of P53 in other events has not been documented, there is some evidence that loss of P53 function is an indicator of stimulation of tumour growth by tumour-produced NO. In a series of human tumour-cell lines expressing wild-type *P53*, mutant *P53*, or no *P53*, when

stimulated to produce NO by transduction of iNOS, *in vivo* growth characteristics of tumour xenografts in nude mice was dictated by their *P53* status: those with wild-type *P53* had delayed growth, whereas those with mutated or missing *P53* showed accelerated growth, expression of vascular endothelial growth factor, and increased tumour angiogenesis.[54] High apopotic indices of tumour cells *in vivo*, taken in isolation, may not necessarily indicate a favourable outcome. In the case of breast cancer, high NOS expression is positively correlated with high apoptotic indices, increased microvascular density, and poor prognosis.[25]

NO and angiogenesis

Angiogenesis is defined as the process of development of new blood vessels from pre-existing vessels, as opposed to vasculogenesis, which is the process of *de novo* formation of blood vessels from vasculogenic precursor cells in the embryo. Angiogenesis occurs normally in the ovary and the uterus during the reproductive cycle, in the pregnant uterus, and as part of wound healing. It also occurs under various pathological conditions, including the growth of solid tumours, which require angiogenesis for growth beyond a diameter of 1 mm.[55] Much evidence suggests a stimulatory role of NO in angiogenesis in various conditions including tumour growth. For example, NO donors stimulate proliferation and migration of endothelial cells *in vitro*. Angiogenesis induced in rabbit cornea with the vasoactive molecules substance P and prostaglandin E was blocked with NOS inhibition.[56] Similarly, NOS inhibitors reduced neovascularisation in gastric ulcers induced with acetic acid in rats[57] and human squamous-cell-carcinoma xenografts in rabbit cornea.[29] An angiogenesis-promoting role of tumour-derived NO was suggested by increased vascularity and growth of a colon-cancer cell line in nude mice after iNOS transduction.[31] *iNOS* knock-out mice showed delayed wound closure, which was corrected by *iNOS* gene transfection.[58] Similarly, *eNOS* knock-out mice showed impaired angiogenesis in ischaemic hind limb,[59] as well as impaired wound healing.[60] Dietary supplementation of L-arginine, the substrate for NOS, improved angiogenesis in ischaemic hind limb.[59]

NO is the final mediator of angiogenesis stimulated by vascular endothelial growth factor (VEGF),[61] the major factor implicated in angiogenesis of many human tumours.[62] Functional eNOS is absolutely required for endothelial-cell migration induced by this growth factor. Impedance analysis of endothelial cells has shown that NO mediates spontaneous micromotion (podokinesis) even in stationary cells, and VEGF transforms this scalar motion to vectorial motion.[63] Stimulation of eNOS by VEGF can be mediated by several mechanisms. The first is upregulation of eNOS mRNA and protein.[64] Second, eNOS can be activated through increased association with heat-shock protein 90,[65] activation of phosphatidylinositol-3OH-kinase/protein kinase B, leading to phosphorylation of eNOS,[66] and activation of MAP kinase/phospholipase C-γ leading to increased phosphatidylinositol triphosphate and intracellular calcium ions.[67]

A positive association has been made between loss of P53 function and vascularity of iNOS-transduced human tumour xenografts.[54] Conversely, wild-type *P53* is believed to be a negative regulator of angiogenesis by downregulating VEGF[68] and promoting the effects of thrombospondin 1, a potent inhibitor of angiogenesis.[69] *P53* mutation in head and neck cancer may be instrumental in iNOS upregulation, which may in turn promote angiogenesis,[18] a hypothesis supported by upregulation of iNOS in cancer-prone *P53* knock-out mice.[70]A promoting role of NO in tumour-induced angiogenesis can be indirect – ie angiogenesis

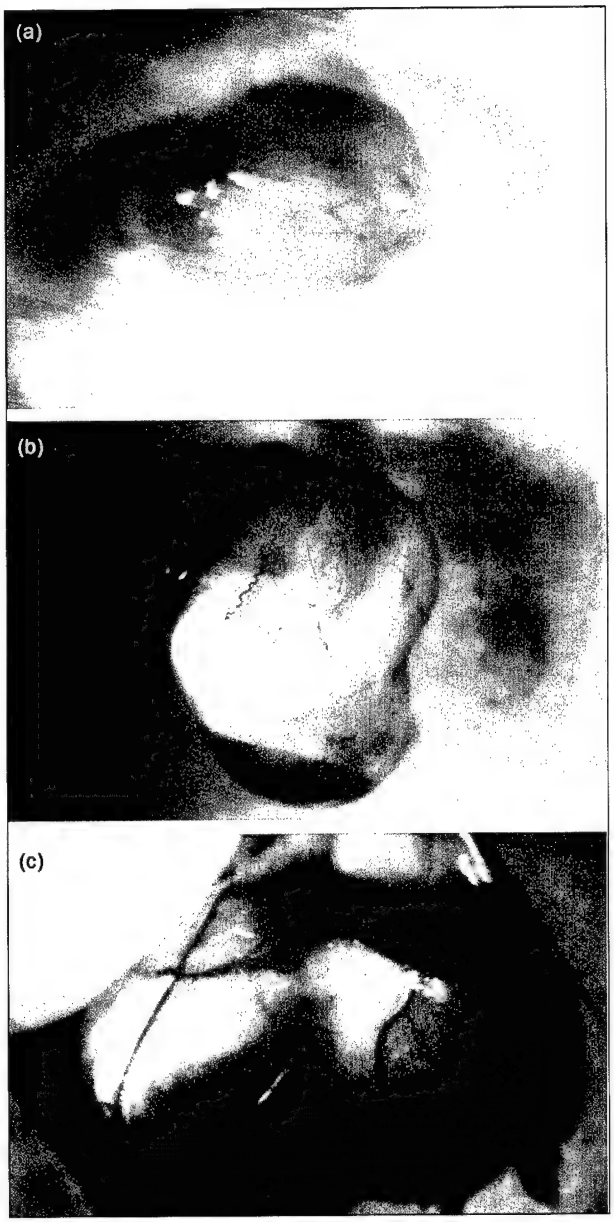

Figure 3. A comparison of the gross morphology of matrigel implants utilised in tumour-induced angiogenesis assay,[35] retrieved at 2 weeks after implantation of matrigel alone (a) or matrigel inclusive of tumour cells (b,c) in mice which received L-NAME therapy (b) or D-NAME (control; inactive enantiomer of L-NAME) therapy (c) via osmotic minipumps until sacrifice. Tumour-exclusive implants (a) were translucent and avascular, and unaffected by L-NAME or D-NAME treatment. Tumour-inclusive implants were smaller and less vascular in L-NAME (b) than D-NAME (c) treated mice. (Reproduced with permission from Am J Pathol, the official journal of the American Society for Investigative Pathology).

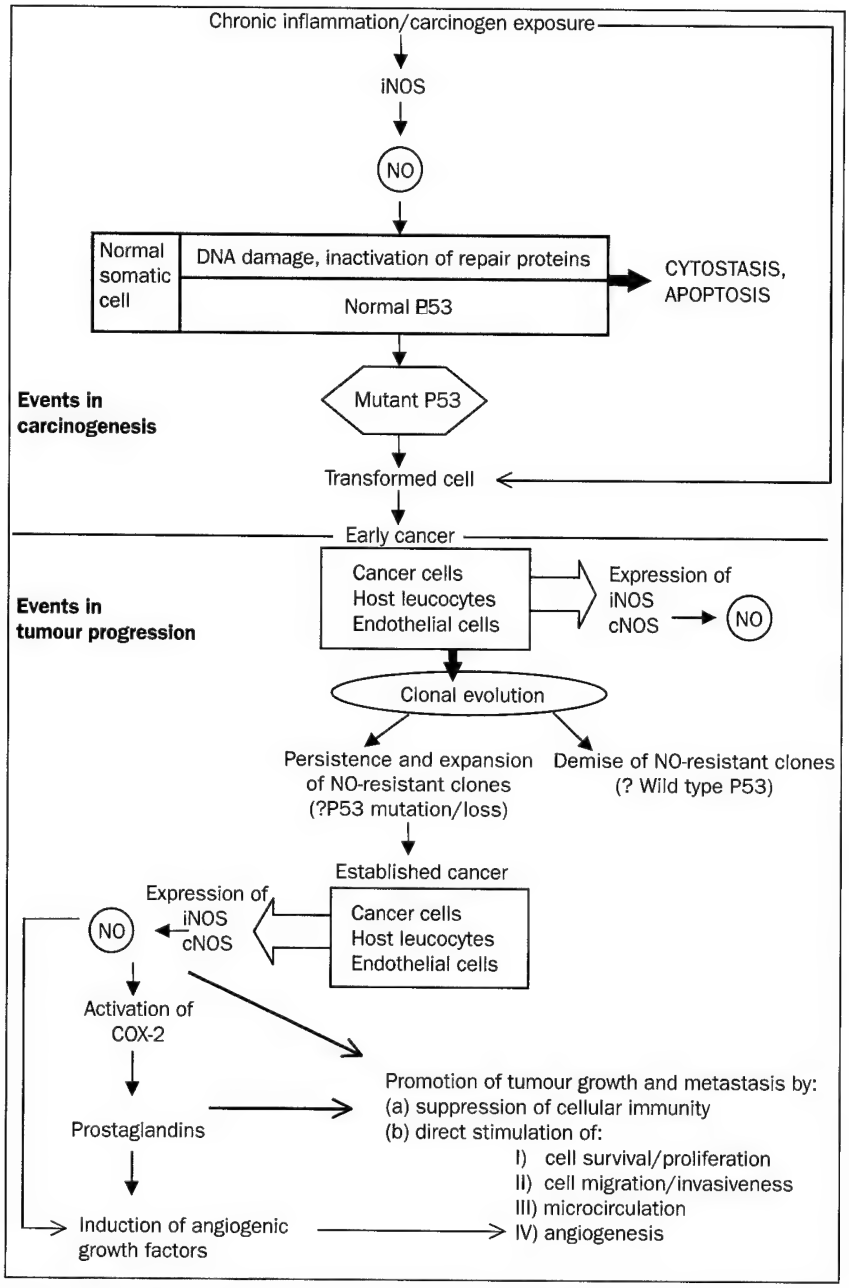

Figure 4. Role of NO (drawn within a circle to include its reactive products) in carcinogenesis and tumour progression.

mediated by VEGF – in which NO released by activation of eNOS in endothelial cells acts as the final mediator, or direct, in which there may be additional NO production by tumour cells or tumour-associated cells. iNOS induction[30] and transduction[31] increase tumour growth and vascularity *in vivo*. iNOS-expressing human squamous-cell carcinomas were highly vasculogenic in rabbit cornea.[29] In a novel tumour-induced angiogenesis assay that uses subcutaneous implants of growth-factor-reduced matrigel including tumour cells,[35] a highly metastatic mammary-tumour cell line with strong eNOS expression was highly angiogenic, and the angiogenic ability was greatly reduced with L-NAME therapy in mice.[35] Gross morphology of matrigel implants in this angiogenesis assay is shown in Figure 4. This model of tumour-induced angiogenesis is highly adaptable to human tumour-cell xenografts in nude mice and seems to be more appropriate than other models of angiogenesis for testing antiangiogenic drugs. For example, in the *in vitro* model of endothelial-cell culture, reconstitution of all the cellular constituents of the tumour microenvironment is difficult. The chick chorioallantoic membrane assay cannot distinguish between angiogenesis and vasculogenesis. The rat or rabbit cornea assay with human cells cannot exclude xenograft-induced angiogenesis.[35]

Role of NO in host antitumour immunity

Cells of the host immune system able to mount antitumour defence are activated natural killer cells, T cells, and activated macrophages. Of the effector molecules released by these cells that can inflict damage on tumour target cells, NO is important primarily for cytotoxic macrophages[3] and, to a lesser extent, for natural killer cells.[71] However, at the same time, there is strong evidence to suggest that macrophage-derived NO inhibits antitumour responses of cytotoxic T lymphocytes *in vivo*, probably through NO-mediated cytostatic effects on T-cell proliferation.[72] Similarly interleukin-2-induced generation of lymphokine-activated killer cells *in vitro* as well as *in vivo* is compromised by concomitant induction of NO production, which can be abrogated with NOS inhibitors.[44] This effect may be due, at least partly, to NO-mediated precursor-cell apoptosis during induction of these cells.[73] Finally, cytotoxic function of NO-producing macrophages is also compromised by NO, which eventually leads to their apoptosis.[3] Thus, high NO concentrations in the effector cell microenvironment are detrimental for antitumour function of all effector-cell lineages.

Role of COX-2 in NO-mediated stimulation of tumour progression

NO can also promote tumour progression by stimulating COX-2, the rate-limiting enzyme for high-output production of prostanoids. Concentrations of both NO and prostaglandins have been positively associated with inflammation and with tumour progression. Several studies

Search strategy and selection criteria

Referred papers were identified by MEDLINE search through the PubMed database (1986 – 2000) by combining the key word 'nitric oxide' with the keywords: carcinogenesis, mutagenesis, apoptosis and tumour, endothelial cell and tumour, angiogenesis. Further search was made by combining the words 'nitric oxide and tumour' for the following journals: *Cancer Research, Journal of the National Cancer Institute, British Journal of Cancer, Journal of Biological Chemistry, Journal of Clinical Investigation, FASEB Journal, Proceedings of the National Academy of Science USA*. Additional papers were identified by searching of references through retrieved papers. Papers were selected on the basis of the best available evidence for each specific question discussed. To limit the number of references, review articles or the latest publications in a series of articles from the same laboratory were given preference. Only papers published in English were included.

in inflammation models have shown reciprocal interactions between NOS and cyclo-oxygenase pathways, most of which show stimulation of COX-2 by NO, although inhibition has also been documented in some cases. Conversely, NOS enzymes can also be modulated by cyclo-oxygenase products.[74] High prostaglandin production by tumours results from upregulation of COX-2, which has been documented in many human cancers, including those of the colon, stomach, breast, head and neck, and pancreas.[75] Chemotherapeutic or chemopreventive drugs able to inhibit both COX-2 and iNOS may become important adjuvants for inclusion in therapeutic protocols. Figure 4 summarises the roles of NO in the key events during the process of carcinogenesis and tumour progression.

Conclusion

The role of NO in tumour biology is complex, because it has both facilitatory and inhibitory roles in cellular processes depending on the conditions, such as the genetic make-up of the cells, the local concentration of NO, and the presence of other regulators such as NO scavengers. Nevertheless, we now have a better knowledge of how these variables regulate NO action on cellular processes responsible for carcinogenesis and tumour progression. Some of the molecular pathways have been identified. NO resistance of tumour cells seems to be associated with NO dependence. This association needs further validation. Further studies of molecular and genetic markers that may determine susceptibility or resistance of tumour cells to NO would greatly facilitate development of newer therapeutic approaches to use NO donors and scavengers and NOS inhibitors depending on the circumstances.

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ROLE OF NITRIC OXIDE IN TUMOUR PROGRESSION WITH SPECIAL REFERENCE TO A MURINE BREAST CANCER MODEL[1]

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ABSTRACT

Nitric oxide (NO) is a potent bioactive molecule produced in the presence of NO synthase (NOS) enzymes, mediating numerous physiological functions under constitutive conditions. Sustained overproduction of NO (and NO-reaction products), typically under inductive conditions, can lead to cell cycle arrest and cellular apoptosis. Furthermore, carcinogenesis may result from mutational events following DNA damage and hindrance to DNA repair (e.g., mutation of tumour-suppressor gene p53, demonstrated in colonic carcinogenesis). In a large majority of human and experimental tumours, tumour-derived NO appears to stimulate tumour progression. However, for a minority of tumours, the opposite has been reported. This apparent discrepancy is likely explained by differential sensitivity of tumour cells to NO-mediated cytostasis or apoptosis, and emergence of NO-resistant and NO-dependent clones. NO-resistance was found to be mediated by p53 inactivation, and upregulation of cyclo-oxygenase-2 and heat shock protein 70. In a murine mammary tumour model, NO promoted tumour growth and metastasis by enhancing invasive, angiogenic and migratory capacities of tumour cells. Invasion-stimulation follows altered balance of matrix metalloproteases and their inhibitors; migration-stimulation follows activation of guanylate cyclase and MAP kinase pathways. Selective NOS inhibitors may have a therapeutic role in certain cancers.

Key Words: nitric oxide, carcinogenesis, invasion, metastasis, angiogenesis

Furchgott and Zawadzki reported that acetylcholine-induced relaxation of smooth muscle surrounding the endothelium occurred only if the endothelium was intact, implying that a signal-inducing molecule was being released by endothelial cells and acting on smooth muscle cells (Furchgott and Zawadzki, 1980). They called the postulated molecule endothelium-derived relaxing factor (EDRF) (Cherry et al., 1982). The discovery in 1987 that nitric oxide (NO) and EDRF were the same entity (Ignarro et al., 1987) prompted a rapid explosion of information of the physiological and pathological roles of this molecule. In 1992, NO was named 'Molecule of the Year' by the journal Science, and in 1998 the Nobel prize was awarded to Furchgott, Ignarro and Murad for their fundamental discoveries of the physiological roles of NO.

NO is the end product of a 5 electron oxidation in which the amino acid L-arginine is converted to L-citrulline, with the reactive NO as a by-product. This reaction is catalyzed by a family of enzymes, the nitric oxide synthases (NOS). Molecular cloning and sequence analysis have revealed the existence of three distinct isoforms of NOS. In general, neuronal (n) and endothelial (e) NOS isoforms (NOS1 and NOS3, respectively) are expressed constitutively; they are expressed continuously and generate NO under conditions where intracellular calcium is increased and calmodulin is activated. When expressed, these isoforms result in steady production of small amounts of NO. In contrast, exposure of cells (e.g., macrophages and hepatocytes) to inflammatory cytokines and/or bacterial products causes expression of the inducible (i) isoform (NOS2). NOS2 is calcium/calmodulin independent, and when expressed, generates large amounts of NO; this high NO production may be sustained for a long period of time (Reviewed by Moncada and Higgs, 1993; Knowles and Moncada, 1994; Geller and Billiar, 1998).

A clear role for NO as a critical mediator of numerous physiological functions has emerged. When constitutively expressed, NO produced at low levels mediates its physiological functions, such as vasodilation, smooth muscle relaxation, inhibition of platelet aggregation, and regulation of neurotransmission. NO is also recognized as an important intercellular messenger that can influence important signaling pathways and modulate gene expression in target cells. Under inductive conditions, high levels of NO produced by macrophages and other effector cells can mediate antibacterial and antitumour functions. However, chronic induction of NOS2 may contribute to many pathological processes including inflammation-mediated tissue damage and carcinogenesis (Reviewed by Moncada and Higgs, 1993; Knowles and Moncada, 1994; Lala and Chakraborty, 2001).

The present article will briefly review the role of NO in carcinogenesis and tumour progression with special emphasis on studies in our laboratory using a murine model of mammary adenocarcinoma.

CHEMICAL REACTIVITY AND CARCINOGENIC ROLE OF NO

NO has an unpaired electron, hence is a free radical ($NO^{\cdot}$), highly reactive, and capable of reacting with other free radicals, molecular oxygen and heavy metals. Direct biological effects of NO comprise those chemical reactions in which NO reacts directly with a biological target, and mostly in conditions where constitutive NOS isoforms generate low levels of NO (Wink et al., 1998). For example,

low levels of NO may react directly with heme-containing proteins such as guanylate cyclase, oxyhemoglobin and cytochrome P450, mediating the neuromodulatory and vasodilatory effects of NOS1 and NOS3, respectively. Indirect effects of NO are those chemical reactions mediated by reactive NO species (RNOs), which are formed by reaction of NO with either oxygen or superoxide. These reactions require high local concentrations of NO, implicating NOS2 as their biological source of NO (Wink et al., 1998).

Chronic exposure of cells to high NO concentrations may lead to genotoxicity, mutagenesis, and by extrapolation, carcinogenesis. NO may mediate DNA damage through several mechanisms: 1) formation of carcinogenic nitrosamines, 2) RNOs-mediated DNA damage (e.g., deamination and transition and/or transversions of nucleic acid bases), and 3) inactivation of DNA-repair proteins (e.g., alkyltransferase and DNA ligase). In addition, some RNOs (e.g., peroxynitrite) result in DNA strand breaks (deRojas-Walker et al., 1995; King et al., 1992; Salgo et al., 1995). DNA damage and mutations induced by NO and its reactive metabolites have been investigated in a variety of experimental conditions, both *in vitro* and *in vivo*.

Role of NO in Carcinogenesis—in vitro evidence: Substantial *in vitro* evidence suggests a role of NO in carcinogenesis. For example, NOS2-expressing macrophages contained deamination and oxidation base products; NOS inhibition reduced formation of these products (deRojas-Walker et al., 1995). Exposure of target DNA to NO gas results in a variety of lesions. For example, NO gas induced C to T transition mutations in *S. typhimurium* (Wink et al., 1991). DNA deamination and strand breaks were evident in human TK6 lymphoblastoid cells following NO gas exposure (Nguyen et al., 1992). Likewise, treatment of Chinese hamster ovary (CHO) cells with NO gas or peroxynitrite resulted in single-strand breaks (Tamir et al., 1996). The mutation pattern induced by NO by reaction of plasmid DNA *in vitro* was examined in the supF gene of the pSP189 shuttle vector replicated in either human Ad293 or *E. coli* MBM7070 cells (Routlege et al., 1993). Relative to spontaneous background mutations, NO-induced mutations were 44-fold and 15-fold higher in human and bacterial cells, respectively. The predominant mutations were A:T to G:C and G:C to A:T transitions (Routlege et al., 1993). Mutagenicity of peroxynitrite, a powerful oxidant formed through reaction of NO with superoxide, was examined in the supF gene of the pSP189 shuttle vector replicated in human AD293 or *E. coli* MBL50 cells. *In vitro* exposure to peroxynitrite induced mutations in human and bacterial cells. In both systems, predominant mutations occurred at G:C base pairs, predominantly involving G:C to T:A transversions, although G:C to C:G transversions and G:C to A:T transitions were also observed (Juedes and Wogan, 1995).

Experimental evidence suggests that NO promotes carcinogenesis by inhibiting DNA repair of cells. Arsenite (a human carcinogen) inhibits UV-induced DNA repair, and NO plays a role in the toxicity of arsenite. Bau and colleagues showed involvement of NO in arsenite-induced inhibition of pyrimidine dimer excision in CHO cells (Bau et al., 2001). Arsenite exposure increased NO production in CHO

cells. Furthermore NOS donors inhibited UV-DNA repair, and inhibition of UV-induced DNA repair by arsenite was suppressed with NOS inhibitors (Bau et al., 2001).

Role of NO in Carcinogenesis—in vivo evidence: *In vivo* studies further support the role of NO in carcinogenesis. Because NO has mutagenic properties, long-term exposure of cells to NOS2-derived NO during chronic inflammation may participate in carcinogenesis. For example, using a model of inflammation in which transgenic mice carry the Lac-Z gene, transplanted B-cell lymphoma cells locating to the spleen stimulated macrophage-derived NO production, resulting in DNA lesions in Lac-Z promoters. DNA lesions were alleviated with NOS inhibitors (Gal and Wogan, 1996). A causal role of *Helicobacter pylori* infection in gastric carcinogenesis has been established, in which NO may play a role; H. pylori infection induces NOS2 expression, and sustained production of NO by infiltrating host macrophages and polymorphonuclear leukocytes (Mannick et al., 1996; Hahm et al., 1997). NO and reactive metabolites then mediate DNA damage, cell cycle arrest and apoptosis (Hahm et al., 1997); mutagenesis may follow DNA damage. Expression of NOS2 was higher in nontumourous colon tissues obtained from cancer-prone ulcerative colitis patients relative to those obtained from healthy, control subjects (Hussain et al., 2000), and it was thought that NOS2-derived NO and reactive products, generated during chronic inflammation contribute to DNA damage (Hussain et al., 2000). Taken together, *in vitro* and *in vivo* evidence presented above indicate that DNA damage and somatic mutation mediated by NO and its reactive products play an important role in carcinogenesis.

ROLE OF NITRIC OXIDE IN TUMOUR PROGRESSION

Much scientific research has focused on the role of NO in tumour progression; although two apparently conflicting views exist, overall an overwhelming amount of clinical and experimental evidence supports a positive association between NO and tumour progression. For example, the level of NOS protein and/or activity in the tumour has been positively correlated with the degree of malignancy for tumours of the human reproductive tract (Thomsen et al., 1994), breast (Thomsen et al., 1995; Dueñas-Gonzalez et al., 1997), and central nervous system (Cobbs et al., 1995). In a majority of gastric carcinomas, NOS2 was detected in stromal elements, and NOS3 was detected in the tumour vasculature (Thomsen and Miles, 1998). NOS3 has been detected in cancer cells of head and neck squamous cell carcinoma (Bentz et al., 1999), salivary carcinoma (Bentz et al., 1998) and endometrial carcinoma (Bentz et al., 1997). NOS2 expression was higher in prostatic carcinomas relative to benign prostatic hyperplastic (Klotz et al., 1998) or control, noncancerous prostates (Uotila et al., 2001). Similarly, relative to normal healthy control tissue, total NOS activity was higher in carcinomas of the larynx, oropharynx, oral cavity (Gallo et al., 1998), and adenocarcinomas of the lung (Fujimoto et al., 1997). Finally, NOS2 protein and/or NOS2 mRNA expression in primary tumours have been positively correlated with lymph node metastases resulting from oral squamous cell carcinomas (Brennan et al., 2001), breast cancer (Dueñas-Gonzalas et al., 1997) and head and neck cancer (Gallo et al., 1998).

Experimental tumour models have provided direct evidence for a promoting role of NO in tumour progression. Treatment with the NOS inhibitor N^G-nitro-L-arginine (L-NAME) reduced NO production and tumour growth in a rat adenocarcinoma model (Kennovin et al., 1994) Induction of NOS2 with lipopolysaccharide (LPS) and interferon (IFN-γ) in EMT-6 murine mammary tumour cells stimulated tumour growth and metastasis *in vivo* (Edwards et al., 1996). Furthermore, NOS2 transduction in a human colon adenocarcinoma cell line resulted in enhanced tumour growth and vascularity when transplanted in nude mice (Jenkins et al., 1995); this effect was inhibited by treatment with a selective NOS2 inhibitor, 1400W (Thomsen et al., 1997).

Primary tumour growth of B16-F1 melanoma cells and vascular-derived endothelial growth factor $(VEGF)_{165}$ mRNA expression within tumours was reduced in $NOS2^{-/-}$ relative to $NOS2^{+/+}$ mice (Konopka et al., 2001). Likewise, Shi et al., (2000) found that B16-BL6 melanoma cells produced fewer and smaller metastases in $NOS2^{-/-}$ mice relative to $NOS2^{+/+}$ mice (Shi et al., 2000). Therefore, host expression of NOS2 appears to contribute to melanoma growth *in vivo*, perhaps by regulating the amount and availability of VEGF.

Inverse Association between NO and Tumour Progression: Not all the literature is consistent with NO increasing tumour growth; a number of studies reported an inverse association between NO and tumour progression. For example, the levels of NOS enzymes and NOS activity declined during the transition of human colonic mucosa to polyps, and then to carcinomas (Chhatwal et al., 1994). However, a later study reported peak levels of NOS activity occurred in adenomatous polyp stage of tumours; increased NOS activity was thought to promote mutational events and angiogenesis, before the transition of adenomas into carcinomas (Ambs et al., 1998). In a murine melanoma cell line, NOS activity was inversely correlated with capacity for metastasis (Dong et al., 1994). When genetically transduced to overexpress NOS2, the melanoma cells (Dong et al., 1994), as well as renal carcinoma cells (Juang et al., 1998), lost their tumorigenic and metastatic capacities, a result of NO-mediated tumour apoptosis. However, NOS2 overexpression in transduced cells was detrimental to cellular survival *in vitro*, necessitating maintenance with NOS inhibitors in culture, suggesting that this laboratory model may not reflect natural events *in vivo*.

Conflicting Roles of NO in Tumour Progression? Many factors contribute to the etiology of cancer, with incipient oncogenic changes occurring long before the appearance of clinically relevant lesions. Apparently, NO plays a complex and sometimes contradictory role in carcinogenesis and tumour progression. Complexities arise because all tumour cells are not equally sensitive to NO-mediated cytotoxicity, and, numerous factors contribute to tumour cells sensitivity or resistance to NO. Two factors appear critically important in determining sensitivity/resistance to NO: 1) NO concentration in the tumour cell microenvironment, and 2) genetic constitution of tumour cells. High NO concentrations have cytostatic and cytotoxic effects, and may result in DNA damage, the initial step in carcinogenesis. In

contrast, low to moderate concentrations of NO may enhance tumour growth, affecting tumour functions such as angiogenesis, invasion and migration.

During clonal evolution of tumours in the presence of high NO concentrations, NO-sensitive cells may be deleted and NO-resistant cells emerge, due at least partly, to mutational events mediated by NO (Lala and Oruvevic, 1998). An important event contributing to evolutionary development of tumours, and that may lead to NO resistance, is mutation of the p53 tumour suppressor gene. A clear role for mutated p53 in carcinogenesis and tumour progression, in which NO contributes, is emerging.

p53

Tumour formation and growth is characterized by uncontrolled cellular proliferation, usually the result of multiple genetic and epigenetic insults to the cell, and involving proto-oncogenes and tumour suppressor genes. One frequently altered tumour suppressor gene believed to play a pivotal role in preventing the uncontrolled growth characteristic of cancer is p53, a gene found to be mutated or deleted in about 50% of all spontaneously arising tumours (Baker et al., 1989, 1990; Caron de Fromentel and Soussi, 1992; Hollstein et al., 1994).

Wild-type tumour suppressor protein p53 is known as a critical regulator of the cellular response to DNA damage (Messmer and Brüne, 1995). In normal cells, p53 protein levels are low, a result of its rapid proteolytic degradation mediated by MDM2 and JNK. However, DNA damage causes post transcriptional p53 stabilization, prolongs its half-life, and results in p53 accumulation. The target genes of p53 affect cell cycle arrest (e.g., $p21^{waf1}$, 14-3-3σ and GADD45) and apoptosis (e.g., Bax, Fas/APO-1, killer/DR5 and p53AIP1). Therefore, p53 activation blocks cellular proliferation by promoting 1) G_1 cell cycle arrest, allowing damaged DNA repair, or in the case of severe DNA damage, 2) induction of apoptosis, thus deleting cells unable to repair damaged DNA.

NITRIC OXIDE AND p53

A fascinating area of research concerns the interplay between NO and p53 expression. A NO-p53 regulatory loop has been proposed, which provides a mechanism by which p53 protects cells from potential NO-mediated DNA damage; increased production of NO induces p53 protein upregulation, which then downregulates NOS2 expression. However, chronic NO exposure and p53 transcription may lead to mutated/inactive forms of the tumour suppressor gene (Calmels et al., 1997). Following p53 gene mutation, the negative feedback loop fails, and p53 mutated cells produce NO unchecked.

Substantial experimental evidence indicates that exposure of cells to high concentrations of NO causes DNA damage. The initial cellular response to NO-mediated damage, to maintain genome integrity, is p53 accumulation and activation, resulting in cell cycle arrest or apoptosis (Forrester et al., 1996; Ho et al., 1996; Meβmer et al., 1994). In turn, p53 accumulation downregulates NOS expression by binding to a promoter site on the NOS2 gene, thus limiting the extent of NO-mediated damage.

Supporting this, wild-type p53 protein expression in numerous human tumour cell lines, and murine fibroblasts, was associated with decreased NOS2 expression (Ambs et al., 1997).

NO-p53 Interaction in Carcinogenesis—NO inactivates p53: Compelling evidence suggests that NO may promote carcinogenesis by inactivating the tumour-suppressor oncoprotein p53 (Murata et al., 1997). NO induces a conformational change of wild-type p53, impairing its DNA-binding activity *in vitro* and rendering p53 inactive (Calmels et al., 1997; Chazotte-Aubert et al., 2000). Inactive or mutated p53 protein does not alter NOS2 expression; dysregulated NOS activity results. Supporting this notion, NOS2 expression was increased in p53 knockout mice, a condition that causes early death from formation of multiple tumours (Ambs et al., 1998).

NO-p53 Interaction in Carcinogenesis—clinical evidence: Clinical research supports an interaction between NOS2 and mutated p53 in eliciting carcinogenesis and/or tumour progression. NOS2 expression and p53 mutation frequencies were higher in nontumourous colon tissues obtained from cancer-prone ulcerative colitis patients relative to those obtained from healthy, control subjects (Hussain et al., 2000). Mutation frequencies were also higher in inflamed lesional tissue relative to nonlesional regions of the same colon. These data suggest an inflammation-associated oxidative stress in the etiology of the p53 mutations present in the cancer-prone condition, ulcerative colitis. (Hussain et al., 2000). Examination of 56 cases of oral squamous cell carcinoma demonstrated a positive association between p53 mutation and NOS2 expression (Brennan et al., 2000). Furthermore, a strong positive association between NOS2 expression and frequency of G:C to A:T transitions at CpG dinucleotides (a common p53 mutation) was noted for 118 sporadic colon tumours (Ambs et al., 1999) and 27 carcinomas of the head and neck (Gallo et al., 1999). A positive association between NOS2 expression and p53 mutation was also observed in 30 early-stage adenocarcinomas of the lung; the predominant p53 mutation observed was G:C to T:A transversions (Fujimoto et al., 1998), a mutation induced by benzo(a)pyrene, a carcinogen present in cigarettes (Denissenko et al., 1992). These data suggest a role for NO-p53 interaction in carcinogenesis and/or tumour progression.

In summary, the p53-NO interaction, and its relationship to tumour development and growth appears complex. As a mutagen, NO potentially alters cellular DNA, thus eliciting p53 accumulation and activation, as the cell attempts self-repair. However, NO-induced p53 mutations result in cells unable to arrest/delete upon DNA damage; chromosomal abnormalities can then propagate. Failure of the NO-p53 regulatory loop perpetuates NO-mediated damage. Taken together, it appears that NO production enhances tumour progression by selecting for mutant p53 cells, and against wild-type p53 cells.

Nitric Oxide Sensitive or Resistant? One of the critical genetic determinants of NO-mediated susceptibility relates to the functional status of p53; functional p53 confers NO-susceptibility, whereas

inactive/mutant p53 confers NO-resistance to tumour cells. NOS2-transfected tumour cells expressing wild-type functional p53 were susceptible to NO-mediated cytostasis, an effect attributed to NO-mediated accumulation of p53 protein (Forrester et al., 1996). However, tumour cells in which the p53 gene is deleted or mutated were resistant to NO-mediated cytotoxicity and apoptosis (Brüne et al., 1996; Ho et al., 1996). In the presence of endogenous NOS2, p53-mutated tumour cells grew faster and were more vascular than those expressing wild-type p53 (Ambs et al., 1998). In support of the requirement for functional p53 for high NO-induced apoptosis, Ho and colleagues showed that human cancer cell lines with wild-type P53 (i.e., Hep G2 and COLO 205) were sensitive, whereas those with mutated (i.e.., HT-29) or missing (i.e., Hep 3B) p53 protein were resistant to NO-mediated cytotoxicity (Ho et al., 1996). Because many human tumours show p53 mutations, these findings, if validated in other tumour lines, may serve as an important marker for NO sensitivity or resistance in human tumours.

NO resistance may be conferred by various other protective mechanisms. For example, activation of cyclo-oxygenase (COX)-2 is involved in conferring NO resistance. When NO-sensitive cells (i.e., RAW264.7 macrophage line) were transfected with a COX-2 expression vector (vonKnethen and Brüne, 1997), or pretreated with small amounts of NO to induce COX-2 through the activation of nuclear factor-κB and activator protein-1 (vonKnethen et al., 1999), the cells became resistant to high doses of NO.

Heat shock protein hsp70 also confers protection against NO-mediated cytotoxicity. Following transfection of human hsp70 into a rat insulinoma cell line, RINm5F, constitutive expression of hsp70 protected cells against NO-induced cell lysis (Bellmann et al., 1996). Human insulin-producing pancreatic beta cells are largely resistant to NO-mediated cytotoxicity (Burkart et al., 2000). To examine the potential of hsp70 in conferring strong natural resistance of human beta cells against NO-induced cytotoxicity, a human beta cell line was established, in which hsp70 expression was selectively suppressed by transfection with antisense-hsp70 mRNA. Relative to hsp70-expressing controls, the antisense-transfected human beta cell line exhibited increased susceptibility to NO-induced cytotoxicity (Burkart et al., 2000). Taken together, these data identify hsp70 as an important defense molecule against NO-mediated injury (Burkart et al., 2000).

NO Resistance Linked to NO Dependence? Irrespective of the mechanisms contributing to NO resistance, NO resistance may lead to NO dependence. This view is supported by the findings of Shi and colleagues (2000), who compared the growth of two murine tumour cell lines, M5076 ovarian sarcoma (NO sensitive) and B16-BL6 melanoma (NO resistant) in wild-type and NOS2 knockout mice; absence of host NOS2 promoted growth and metastasis of the NO sensitive cell line, but impaired growth and metastasis of the NO resistant cell line (Shi et al., 2000).

MECHANISMS UNDERLYING THE STIMULATORY ROLE OF NITRIC OXIDE IN TUMOUR PROGRESSION: LESSONS FROM A MURINE MAMMARY ADENOCARCINOMA MODEL

Our laboratory documented NO-mediated stimulation of tumour growth and metastasis in a murine mammary adenocarcinoma model, which included C3H/HeJ spontaneous mammary tumours and two clonal derivatives of high and low metastatic capacities (C3L5 and C10, respectively) isolated from a single spontaneous tumour. Spontaneously arising primary mammary tumours in C3H/HeJ retired breeder female mice consisted of a heterogeneous mixture of NOS3 protein-expressing and nonexpressing tumour cells. However, a strong and homogeneous expression pattern was observed at metastatic lung sites, suggesting that NOS3 expression provided a selective advantage to metastasis (Lala and Orucevic, 1998; Jadeski et al., 2000). Further evidence supported this notion. The highly metastatic cell line, C3L5, clonally derived from a spontaneously arising mammary tumour, strongly expressed NOS3 protein *in vitro* and *in vivo* and NOS2 protein *in vitro* upon stimulation with LPS and IFN-γ. The weakly metastatic clone, C10, derived from the same parental tumour, expressed low levels of NOS3 protein *in vitro* (Lala and Orucevic, 1998).

Tumours derived from transplanted C3L5 and C10 cell lines differed in NOS3 expression at primary tumour sites; NOS3 expression was higher in primary tumours derived from the highly metastatic C3L5 cell line relative to those derived from the weakly metastatic C10 cell line. However, NOS3 expression did not differ for metastatic colonies derived from C3L5 and C10 tumours; both tumours expressed high levels of NOS3. These data suggested that NOS3 expression was conducive to tumour progression and metastasis (Jadeski et al., 2000). Treatment of C3L5 mammary tumour-transplanted animals with NOS inhibitors N^G-methyl-L-arginine (L-NMMA) or L-NAME reduced both primary tumour growth and spontaneous lung metastases, indicating a causal relationship between NO and tumour growth and metastasis (Orucevic and Lala, 1996*a*, 1996*b*; Lala and Orucevic, 1998).

Key cellular processes that determine primary tumour growth rate are tumour cell proliferation, survival and capacity for angiogenesis; invasiveness and metastases depend on migration and matrix degradation by tumour cells. The underlying mechanisms of NO-mediated promotion of tumour growth and metastasis in this mammary adenocarcinoma tumour model were then examined.

Tumour Cell Proliferation and Invasiveness: Endogenous NO did not alter C3L5 cell proliferation, and the *in vitro* growth rates of C10 and C3L5 cells did not differ. However, the invasive capacities of the two cell lines differed; the C3L5 cell line was more invasive relative to the C10 cell line, consistent with differences in their metastatic capacities *in vivo*. For both cell lines, invasiveness was suppressed with L-NAME treatment and restored with additional exposure of cells to L-arginine, suggesting that endogenously-derived NO promoted invasion, and L-NAME-mediated reduction of tumour cell invasiveness was NO-specific (Jadeski et al., 2000). Furthermore, using the C3L5 cell line, NO-mediated effects on invasive capacity were found to result from a differential regulation of matrix metalloproteases (MMPs) and their natural inhibitors, tissue inhibitors of matrix metalloproteases

(TIMPs). Specifically, under constitutive conditions of NO production by NOS3-expressing C3L5 cells, NO downregulated TIMP-2 and TIMP-3, and under inductive conditions (i.e., in presence of LPS and IFN-γ), MMP-2 was upregulated (Orucevic et al., 1999).

Tumour-induced Angiogenesis: An *in vivo* angiogenesis model using subcutaneous implants of growth-factor reduced Matrigel, inclusive of tumour cells (i.e., C3L5 or C10) was used to evaluate the contributory role of NO in C3L5 mammary tumour-induced angiogenesis (Jadeski and Lala, 1999; Jadeski et al., 2000). Gross morphology of Matrigel implants, retrieved 14 days after experimental transplantation, indicated that tumour-exclusive (i.e., Matrigel only) implants were devoid of blood vessels, whereas tumour-inclusive implants were vascular, validating the capacity of the *in vivo* assay to quantify *tumour-induced* angiogenesis. C3L5 tumour-containing implants were much larger and vascular relative to tumour-exclusive implants, and implants from L-NAME-treated animals appeared less vascular relative to D-NAME-treated animals. Histological evaluation of the implants confirmed these observations, and revealed the presence of three distinct histological regions within the implants in both treatment groups (i.e., L-NAME and D-NAME). Readily apparent were 1) a relatively thin, outer stromal area, from which blood vessels developed and fed into, 2) an inner healthy tumour region, and 3) a more centrally-located necrotic region. Computer-assisted quantification of the three histological regions indicated that stromal tissue and total mass of viable tissue was reduced, and necrotic tissue increased, in implants obtained from L-NAME relative to those obtained from D-NAME-treated animals. Quantification of the incidence of blood vessels, identified on the basis of endothelium-specific marker CD31, revealed a dramatic decline in angiogenic response in L-NAME-treated, relative to D-NAME-treated mice. (Jadeski and Lala, 1999). Vascularity of C3L5-derived implants varied as a function of time; retrieval of the implants at various time points (i.e., day 5, 7, 9, 12 and 14 days after experimental implantation) revealed the decline in tumour vascularization resulting from L-NAME therapy was first detectable at day 12, and more pronounced at day 14 after implantation.

Angiogenic capacities of C3L5 and C10 cell lines varied, consistent with differences in their NO-producing capacities; the angiogenic response was higher in tumours derived from C3L5 cells relative to those derived from C10 cells. Furthermore, in contrast to C3L5-derived tumours, in which the angiogenic response was inhibited with L-NAME treatment, this treatment did not alter angiogenic capacity of low NOS3-expressing C10 cells (Jadeski et al., 2000). Taken together, these data clearly indicate that, in this mammary adenocarcinoma model, endogenous NO plays a stimulatory role in tumour-induced angiogenesis.

Tumour Cell Migration: Despite differences in NOS3 expression and NO production by C3L5 and C10 cells, migratory capacities of the two cell lines did not differ. However, migratory capacities of both cell lines were inhibited in the presence of L-NAME. When L-NAME-treated cells were additionally exposed to L-arginine, the migratory capacities of both cell lines were restored to control levels, indicating that

endogenous NO is required for migration of C3L5 and C10 cell lines, and that the effects of L-NAME on migration are NO-specific (Jadeski et al., 2000).

The intracellular pathways of signal transduction in NO-induced migratory responses in C3L5 mammary adenocarcinoma cell migration have been examined (Jadeski, Chakraborty and Lala, unpublished); two signaling pathways were investigated. Firstly, the NO metabolic pathway, in which NO is produced from the amino acid L-arginine by the action of NOS was examined. NO, in turn, is believed to stimulate guanylate cyclase (GC), mediating production of the important intracellular mediator cyclic guanosine monophosphate (cGMP). Secondly, the mitogen activated protein kinase (MAPK) pathway was examined; this is an important signaling pathway, involving sequential phosphorylations, and is implicated in the regulation of a wide variety of cellular functions including cell proliferation, migration and transmission of oncogenic signals. Activation of the MAPK pathway appears important for migratory response in many cells (Klempke et al., 1998) A component of this pathway, MAPK (ERK), has been shown to activate myosin light chain kinase, which in turn activates the cellular motility apparatus, resulting in cell motility (Figure 1). The roles of the GC and MAPK pathways in NO-mediated effects on C3L5 cell migration was investigated, and a potential link between them elucidated. To achieve these objectives, a variety of agents, either alone or in combination, were used to stimulate [i.e., L-arginine: NOS substrate; sodium nitroprusside (SNP): NO donor; 8-bromoguanosine 3',4'-cyclic monophosphate (8-Br cGMP): cGMP analogue] or inhibit [i.e., L-NAME: NOS inhibitor; 1-H-oxadiaxolo[4,3-a]quinoxalin-1-one (ODQ): guanylase cyclase inhibitor; PD098059: MAPK kinase inhibitor] components of the pathways. The effects of the various agents on *in vitro* C3L5 tumour cell migration and/or ERK phosphorylation were then determined.

NO-mediated promotion of C3L5 cell migration utilizes GC and MAPK pathways: Treatment with ODQ reduced, and 8-Br cGMP increased, migratory capacity of C3L5 cells relative to untreated controls, suggesting that GC was required for C3L5 migratory function. A dose-dependent inhibition of migration was observed following treatment with L-NAME, and as previously observed, the migratory capacity of L-NAME-treated cells additionally exposed to L-arginine was restored to control levels, suggesting that 1) endogenous NO was required for C3L5 migration, and 2) the effects of L-NAME were NO-specific. However, presence of ODQ blocked the migration-restoring effects of L-arginine on L-NAME-treated cells, suggesting that cGMP is required for NO-mediated migration of C3L5 cells. Treatment with the MAPK kinase (MEK) inhibitor PD098059 reduced the migratory capacity of C3L5 tumour cells relative to untreated control cells, suggesting that a functioning MAPK pathway is required for C3L5 migration. Furthermore, PD098059 blocked the migration-restoring effect of L-arginine on L-NAME-treated cells, suggesting that endogenous NO-mediated stimulation of C3L5 cell migration requires a functioning MAPK pathway.

NO-mediated ERK phosphorylation is GC-dependent: Treatment with ODQ reduced, and 8-Br cGMP increased, ERK phosphorylation of C3L5 cells relative to untreated controls, suggesting that cGMP is required for ERK phosphorylation. C3L5 cells treated with L-NAME demonstrated reduced ERK phosphorylation relative to controls, indicating a relationship between endogenous NO and ERK phosphorylation. Additional treatment of L-NAME-treated cells with either L-arginine or SNP increased ERK phosphorylation above control levels, suggesting that endogenous *and* exogenous NO promoted MAPK phosphorylation in C3L5 cells, and that the effects of L-NAME treatment on ERK phosphorylation were indeed NO-specific. Finally, presence of ODQ blocked the ERK phosphorylation-restoring effects of L-arginine on L-NAME-treated cells, suggesting that cGMP is required for endogenous NO-mediated MAPK phosphorylation, and provided a sequential link between the NOS, GC and MAPK pathways in mediating migration of C3L5 cells.

In summary, we have demonstrated three mechanisms of NO-mediated tumour progression: stimulation of cellular invasiveness, migration and angiogenic capacity. Stimulation of C3L5 cell invasiveness resulted from a differential regulation of MMPs and TIMPs. NO enhanced migration of C3L5 cells by activating GC and MAPK pathways. Mechanisms underlying NO-mediated promotion of angiogenesis remain to be investigated. However, NO has been determined to be the downstream mediator of VEGF-induced angiogenesis, where NO-mediated stimulation of endothelial cell proliferation, migration and tube formation was observed (Ziche et al., 1997).

Conclusions: Constitutive NO production is essential for many physiological functions. However, chronic induction of NO can be carcinogenic, partly a consequence of breakdown of p53-mediated cytoprotection via p53 inactivation. NO also promotes tumour progression; NO-resistant cells may emerge during clonal evolution of tumours, and these cells may eventually become NO-dependent. Therefore, host and/or tumour-derived NO promotes tumour progression, as demonstrated in a large majority of spontaneous tumours. A number of cellular processes contribute to NO-mediated tumour promotion: increased survival, stimulation of migratory, invasive and angiogenic capacities of tumour cells; these processes result from NO-mediated signaling events and gene regulation. Figure 2 provides a schematic diagram of the sequence of events in NO-mediated carcinogenesis and tumour progression. Certain human malignancies, in which the stimulatory role of NO in tumour progression is well established (e.g., breast cancer), should benefit from combination therapies inclusive of NOS inhibitors.

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FIGURE LEGENDS

Figure 1. Molecular mechanisms of NO-mediated stimulation of C3L5 tumour cell migration examined in the present study. The NO metabolic pathway: NO is produced from L-arginine, by action of NOS. In turn, NO stimulates GC, mediating production of intracellular mediator cGMP. MAPK pathway: MAPK (ERK) activates myosin light chain kinase, which activates cellular motility apparatus, resulting in cell motility. The roles of the GC and MAPK pathways in NO-mediated effects on C3L5 cell migration was examined, and a potential link between them elucidated using a variety of agents, either alone or in combination, to stimlate or inhibit components of the pathways.

Figure 2. Schematic diagram of the sequence of events in NO-mediated promotion of carcinogenesis and tumour progression. When constitutively expressed (i.e., NOS1, NOS3), low levels of NO mediates its physiologcal functions (e.g., vasodilation, smooth muscle relaxation, inhibition of platelet aggregation and regulation of neurotransmission). Under inductive conditions (i.e., NOS2) high levels of NO may cause DNA damage. As the cell attempts self repair, p53 accumulation and activation result in cell cycle arrest and/or apoptosis. NO-p53 regulatory loop: p53 accumulation downregulates NOS expression, limiting extent of NO-mediated damage. Chronic NO exposure and p53 transcription may lead to mutated/inactive forms of p53: negative feedback loop fails, and NO production is unregulated in p53-mutated cells. NO sensitive/resistant cells: functional p53 confers NO-susceptibility, inactive/mutant p53 confers NO-resistance; other protective mechanisms confer NO-resistance (e.g., activation of COX-2 and upregulation of HSP70). NO resistance may lead to NO dependence. NO then promotes tumour progression by a variety of mechanisms: promotion of tumour cell survival, increased migration, invasion and angiogenesis.

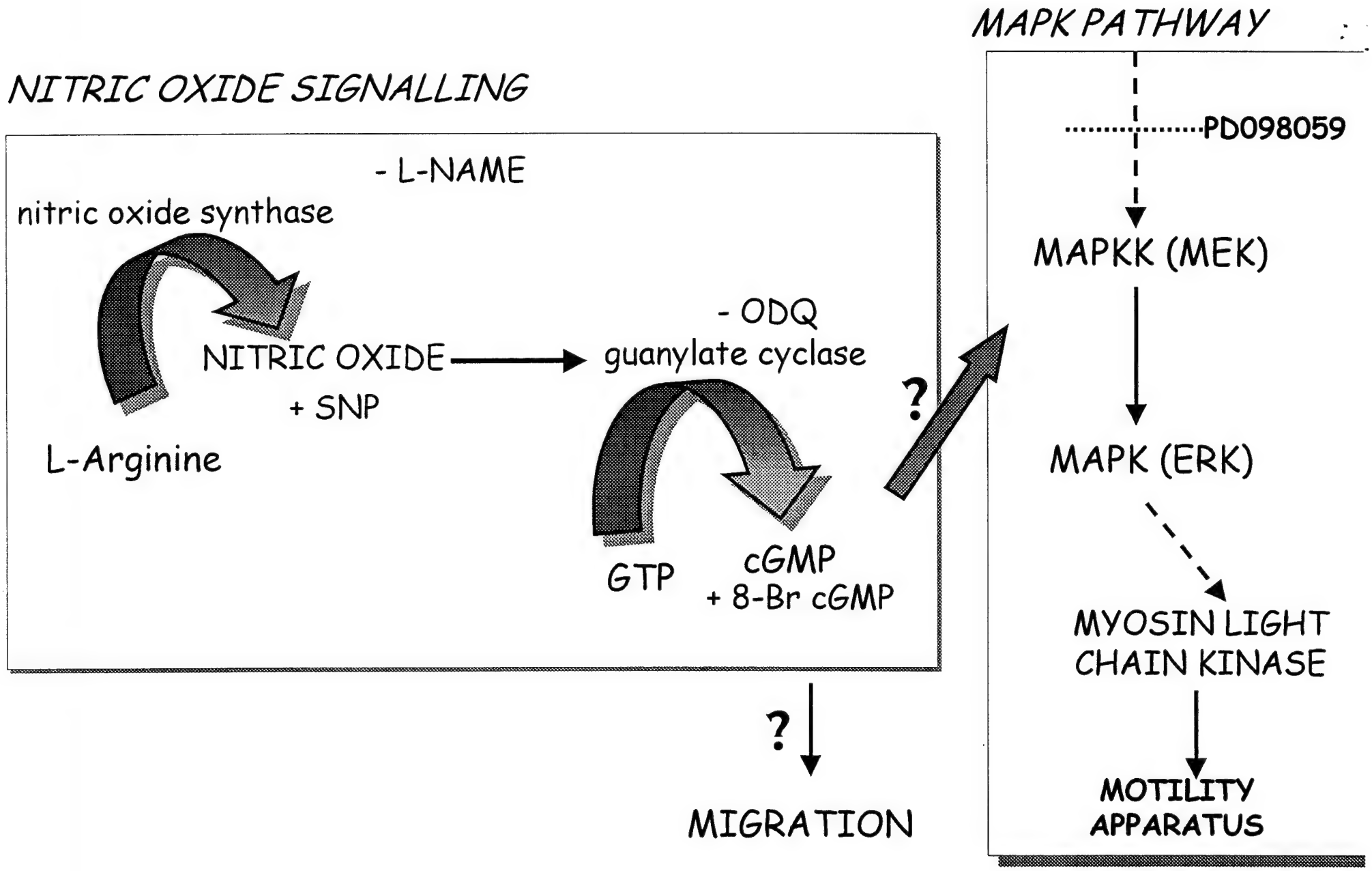
MAPK PATHWAY
NITRIC OXIDE SIGNALLING
PD098059
- L-NAME
nitric oxide synthase
MAPKK (MEK)
- ODQ
guanylate cyclase
NITRIC OXIDE
+ SNP
?
MAPK (ERK)
L-Arginine
GTP
cGMP
+ 8-Br cGMP
MYOSIN LIGHT
CHAIN KINASE
?
MOTILITY
APPARATUS
MIGRATION

FIGURE 1

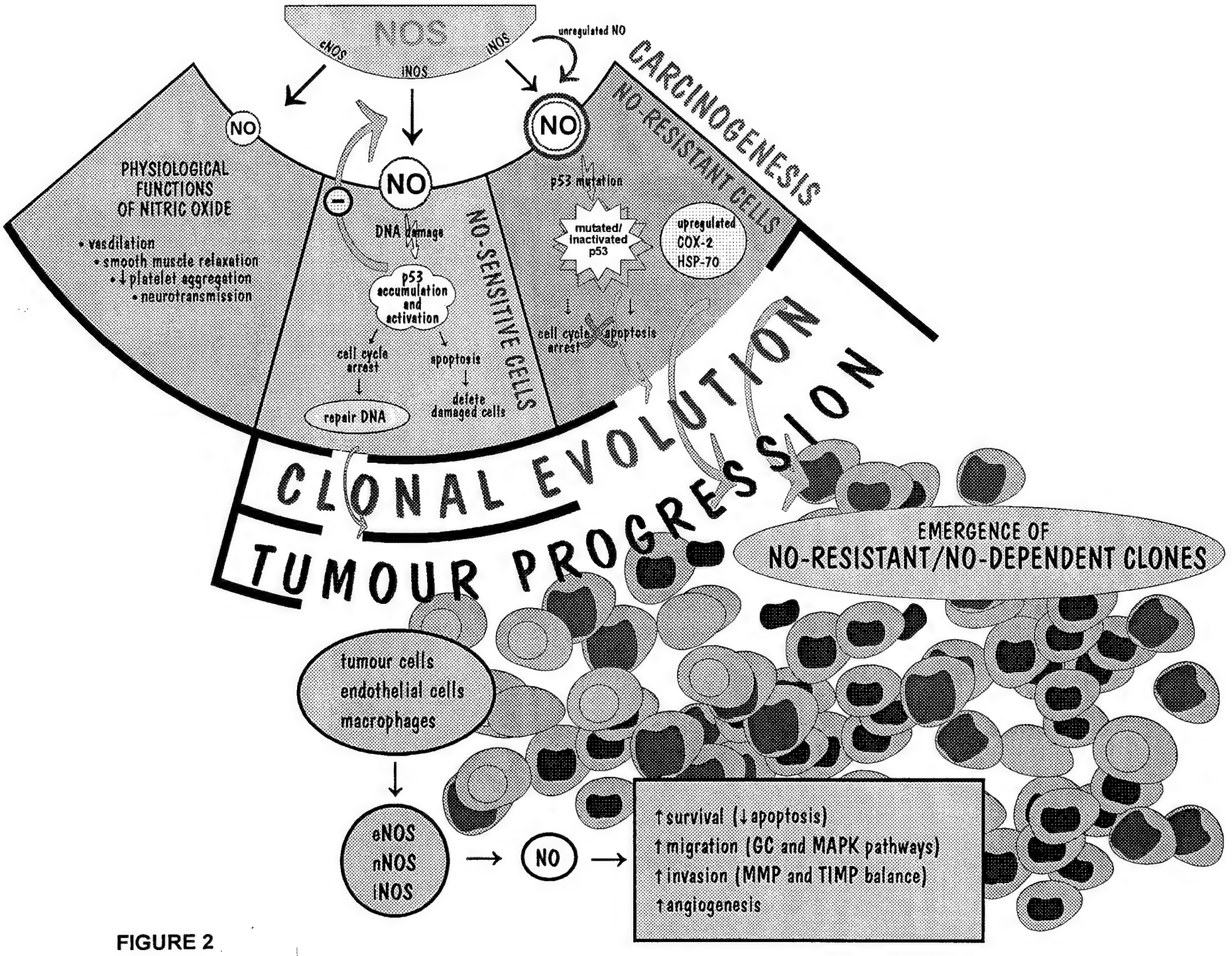
NOS
cNOS
iNOS
iNOS
unregulated NO
NO
NO
NO
PHYSIOLOGICAL FUNCTIONS OF NITRIC OXIDE
• vasdilation
• smooth muscle relaxation
• ↓ platelet aggregation
• neurotransmission
DNA damage
p53 accumulation and activation
cell cycle arrest
repair DNA
apoptosis
delete damaged cells
NO-SENSITIVE CELLS
p53 mutation
mutated/ inactivated p53
upregulated COX-2 HSP-70
cell cycle arrest
apoptosis
NO-RESISTANT CELLS
CARCINOGENESIS
CLONAL EVOLUTION
TUMOUR PROGRESSION
EMERGENCE OF NO-RESISTANT/NO-DEPENDENT CLONES
tumour cells
endothelial cells
macrophages
eNOS
nNOS
iNOS
NO
↑survival (↓apoptosis)
↑migration (GC and MAPK pathways)
↑invasion (MMP and TIMP balance)
↑angiogenesis

FIGURE 2

submitted for publication

NITRIC OXIDE-MEDIATED PROMOTION OF MAMMARY TUMOUR CELL MIGRATION: ROLES OF GUANYLATE CYCLASE AND MAP KINASE PATHWAYS[1]

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ABSTRACT

We found a positive correlation between the expression of endothelial type nitric oxide (NO) synthase (NOS) expression and metastasis in C3H/HeJ spontaneous murine mammary adenocarcinomas, and their clonal derivatives with high or low metastatic capacities. Treatment of mice bearing transplants of a highly metastatic clone, C3L5, with NOS inhibitors L-NAME or NMMA had antitumour and antimetastatic effects, which were shown to result from an abrogation of NO-mediated stimulation of tumour cell migration, invasiveness and angiogenesis. The present study examined intracellular signaling pathways underlying NO-mediated promotion of tumour cell migration, a cellular process essential for invasiveness and metastasis. C3L5 tumour cell migration was reduced in the presence of L-NAME or a guanylate cyclase (GC) inhibitor 1-H-oxadiaxolo[4,3-a]quinoxalin-1-one (ODQ), or their combination. Inhibitory effects of L-NAME were abrogated in the presence of excess L-arginine, the substrate for NOS. Additional exposure of cells to ODQ (i.e., L-NAME, L-arginine and ODQ) inhibited the migration-stimulating effects of L-arginine on L-NAME-treated cells. Conversely, tumour cell migration was increased in the presence of 8-bromoguanosine 3', 5'-cyclic monophosphate (8-Br cGMP: a cGMP analogue). MAPK (ERK1/2) phosphorylation in C3L5 cells was inhibited with L-NAME, and restored rapidly with the additional presence of excess L-arginine, or an NO donor sodium nitroprusside (SNP). Treatment with ODQ inhibited, and 8-Br cGMP stimulated ERK phosphorylation. Combination treatment of L-NAME, L-arginine and ODQ reduced ERK1/2 phoshorylation relative to untreated controls. Finally, PD098059 (MAPKK or MEK inhibitor) blocked basal, as well as NO-stimulated tumour cell migration, and ERK1/2 phosphorylation. These results reveal that signaling for NO-stimulated tumour cell migration involve stimulation of GC, leading to activation of the MAPK pathway.

Key words: nitric oxide, mammary tumour, migration, cyclic GMP, guanylate cyclase, MAP kinase

INTRODUCTION

Nitric oxide (NO), an inorganic free radical gas, is synthesized from the amino acid L-arginine by a group of enzymes, the NO synthases (NOS). At least three isoforms of NOS have been cloned, characterized and localized: endothelial (e) and neuronal (n) NOS isoforms are Ca^{2+}/calmodulin-dependent, and are expressed constitutively in these and other cells. When expressed, constitutive isoforms result in steady production of small amounts of NO. In contrast, the inducible (i) isoform is Ca^{2+}/calmodulin-dependent, and usually induced in the presence of inflammatory cytokines and bacterial products. Under certain conditions, iNOS can also be expressed constitutively in some cells. Constitutively produced NO is an important mediator of numerous physiological functions, such as vasodilation, smooth muscle relaxation, inhibition of platelet aggregation, and regulation of neurotransmission. Under inductive conditions, high levels of NO produced by macrophages and other effector cells can mediate antibacterial and antitumour functions. However, chronic induction of NOS may contribute to many pathological processes including inflammation-associated tissue injury and cancer (reviewed by Moncada and Higgs, 1993; Knowles and Moncada, 1994; Lala and Chakraborty, 2001).

Numerous studies suggest that NO contributes to the progression of certain tumours. The level of NOS protein and/or activity in the tumour have been positively correlated with the degree of malignancy for tumours of the reproductive tract (Thomsen et al., 1994), breast (Thomsen et al., 1995; Dueñas-Gonzalez et al., 1997), and central nervous system (Cobbs et al., 1995). In a majority of gastric carcinomas, iNOS was detected in stromal elements, and eNOS was detected in the tumour vasculature (Thomsen and Miles, 1998). eNOS was detected in cancer cells of head and neck squamous cell carcinoma (Bentz et al., 1999), salivary carcinoma (Bentz et al., 1998) and endometrial carcinoma (Bentz et al., 1997). iNOS expression was higher in prostatic carcinomas relative to benign hyperplastic prostate tissue (Klotz et al., 1998) or control, noncancerous prostates (Uotila et al., 2001). Similarly, relative to normal healthy control tissue, total NOS activity was higher in carcinomas of the larynx, oropharynx and oral cavity (Gallo et al., 1998), and adenocarcinomas of the lung (Fujimoto et al., 1997). Finally, iNOS protein and/or iNOS mRNA expression in primary tumours have been positively correlated with lymph node metastases resulting from oral squamous cell carcinomas (Brennan et al., 2001), breast cancer (Dueñas-Gonzalas et al., 1997), and head and neck cancer (Gallo et al., 1998).

Experimental tumour models have provided direct evidence of a contributory role of NO in tumour progression Using a rat adenocarcinoma model in which cells of the tumour vasculature expressed iNOS, treatment of the host with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) reduced NO production and tumour growth (Kennovin et al., 1994). Induction of iNOS with lipopolysaccharide (LPS) and interferon (IFN-γ) in EMT-6 murine mammary tumour cells stimulated tumour growth and metastasis *in vivo* (Edwards et al., 1996). Furthermore, iNOS transduction in a human colon adenocarcinoma cell line resulted in enhanced tumour growth and vascularity when transplanted into nude mice (Jenkins et al., 1995); this effect was inhibited by treating with a selective iNOS inhibitor, 1400W (Thomsen et al., 1997).

Despite overwhelming evidence indicating a stimulatory role of NO in the progression of human and experimental tumours, a number of studies report an inverse association between NO and tumour progression. For example, in human colonic tumours, an inverse association between NOS expression and tumour progression has been reported (Chhatwal et al., 1994). However, peak NOS activity occurred in colonic adenomas, prior to their transition in to carcinomas; increased NOS was thought to promote mutagenesis/carcinogenesis and angiogenesis (Ambs et al., 1998). Indeed, certain types of p53 mutations were strongly correlated with high NOS activity in these tumours (Ambs et al., 1999). In experimental melanoma (Dong et al., 1994) and kidney cancer models (Juang et al., 1998), iNOS transduction and overexpression reduced tumourigenic and metastatic capacity of tumour cells, a result of to NO-induced tumour apoptosis. The above discrepancy may best be explained by the deletion of NO-sensitive and emergence of NO-resistant clones and NO-dependent, occurring during clonal evolution of many tumours (Lala and Chakraborty, 2001). NO-resistance may result from a variety of mechanisms: p53 mutation (Ambs et al., 1998; Brüne et al., 1996; Ho et al., 1996), and upregulation of cyclo-oxygenase (COX)-2 (vonKnethen and Brüne, 1997) and heat shock protein (HSP)-70 (Bellmann et al., 1996; Burkart et al., 2000). Furthermore, NO-resistance may be associated with NO-dependency (Shi et al., 2000).

Studies in our laboratory have shown that expression of eNOS by tumour cells is positively associated with tumour growth and metastasis in spontaneous mammary adenocarcinomas developing in female C3H/HeJ retired breeder female mice, and two clonal derivatives of high and low metastatic capacities (i.e., C3L5 and C10, respectively), isolated from a single spontaneous tumour (Lala and Orucevic, 1998; Jadeski et al., 2000). Tumour

cells constituting spontaneously-arising mammary tumours were distinctly heterogeneous in eNOS protein expression. However, a strong and homogeneous expression pattern was observed at metastatic lung sites, suggesting that eNOS expression provided a selective advantage to metastases (Lala and Orucevic, 1998; Jadeski et al., 2000). eNOS expression patterns in two clonal derivatives supported this notion. The highly metastatic cell line, C3L5, strongly expressed eNOS protein *in vitro* and *in vivo*, and iNOS protein *in vitro* upon stimulation with LPS and IFN-γ. The weakly metastatic clone, C10, expressed low levels of eNOS protein *in vitro* (Lala and Orucevic, 1998).

Tumours derived from transplanted C3L5 and C10 cell lines differed in eNOS expression at primary tumour sites; eNOS expression was higher in primary tumours derived from the highly metastatic C3L5 cell line relative to those derived from the weakly metastatic C10 cell line. However, metastatic colonies derived from both tumours expressed similarly high levels of eNOS. These data suggested that eNOS expression was conducive to tumour progression and metastasis (Jadeski et al., 2000). A causal relationship between the two events was suggested by our findings that therapy of C3H/HeJ C3L5-bearing mice with NOS inhibitors N^G-methyl-L-arginine (L-NMMA) or L-NAME reduced tumour growth and metastases (Orucevic and Lala, 1996a, 1996b; Lala and Orucevic, 1998). We have identified multiple mechanisms responsible for NO-mediated tumour progression. Tumour-derived NO promoted a) tumour cell invasiveness, by altering the balance between matrix metalloproteases and their inhibitors (Orucevic et al., 1999), b) migratory capacity of tumour cells (Jadeski et al., 2000), and c) angiogenesis (Jadeski and Lala, 1999; Jadeski et al., 2000).

In the present study, we have examined the molecular mechanisms of NO-mediated stimulation of C3L5 tumour cell migration, specifically the intracellular pathways of signal transduction (Figure 1). Firstly, the NO metabolic pathway, in which NO is produced from the amino acid L-arginine, by action of NOS was examined. NO, in turn, is believed to stimulate guanylate cyclase (GC), mediating production of the important intracellular mediator cyclic guanosine monophosphate (cGMP). Secondly, the mitogen activated protein kinase (MAPK) pathway was examined; this is an important signaling pathway, involving sequential phosphorylations, and is implicated in the regulation of a wide variety of cellular functions. A component of this pathway, MAPK (ERK), activates myosin light chain kinase, which in turn activates the cellular motility apparatus, resulting in cell motility (Klempke et al., 1998). The roles of the GC and MAPK pathways in NO-mediated effects on C3L5 cell migration was investigated, and a potential link between them elucidated. To achieve our goals, a variety of

test agents, either alone or in combination, were used to stimulate or inhibit components of the pathways. The effects of these agents on *in vitro* C3L5 tumour cell migration and/or ERK phosphorylation were then determined. Results revealed that endogenous NO promoted migratory capacity of C3L5 tumour cells by activation of guanylate cyclase, followed by MAP kinase pathways.

MATERIALS AND METHODS

Tumour Cell Line

A spontaneously occurring mammary tumour in a female retired breeder C3H/HeJ mouse was the source of a primary transplantable tumour T58 from which a metastatic C3 cell line was cloned. Since the metastatic potential of the C3 line declined over a number of years following repeated in vitro passages (Lala et al., 1986), a highly metastatic C3L5 line was derived by five cycles of repeated *in vivo* selections for spontaneous lung micrometastases following subcutaneous transplantation of C3 cells into C3H/HeJ mice (Lala and Parhar, 1993). The C3L5 cells used in the present study were grown from frozen stock and maintained in RPMI 1640 medium (GIBCO; Burlington, ON, Canada) supplemented with 5% fetal calf serum (GIBCO) and 1% penicillin-streptomycin (Mediatech; Washington, DC) in a humidified incubator, 5% CO_2.

Transwell Migration Assay

Migratory capacity of tumour cells was measured using an *in vitro* transwell migration assay (Gleeson et al., 2001), in which transwells were fitted with millipore membranes (6.5 mm filters, 8 μM pore size; Costar, Toronto, Canada). Test agent examined: L-NAME (NOS inhibitor), L-arginine (NOS substrate), ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one, inhibitor of guanylate cyclase), 8-Br cGMP (8-Bromoguanosine 3'5'-cyclic monophosphate, cGMP analogue) and PD098059 (MAP kinase inhibitor). Concentrations of test agents examined: L-NAME (200 μM - 5 mM); ODQ (10 μM - 750 μM); combination of L-NAME (1 mM) and L-arginine (5mM); combination of L-NAME (1mM), L-arginine (5mM) and ODQ (20 μM); 8-Br cGMP (250 μM); PD098059 (10 μM); or combination of PD098059 (10 μM), L-NAME (1mM) and L-arginine (5mM). C3L5 cells (1.5×10^4 cells/100 μl serum-reduced medium) were placed in the upper chamber of transwells; test agents were added alone, or in combination (as indicated in results), to upper (100 μl) and lower chambers of transwells (800 μl). Chambers were assembled and incubated for 24 or 48 hours (37°C, 5% CO_2). After incubation, cells from the upper surface of millipore membranes were completely removed with gentle swabbing; remaining migrant cells were fixed and stained using Quik® (Dada, Düdingen, Switzerland). Membranes were then rinsed with distilled water, gently cut from transwells and mounted onto glass slides. Cellular migration was determined by counting the number of stained cells on

membranes in 5 randomly selected, non-overlapping fields at 400X magnification under a light microscope (researcher blind to experimental condition).

Western Blot Analysis for ERK1 and ERK2

C3L5 cells were grown to ~80% confluency on tissue culture dishes, serum starved overnight, and exposed to test agents described in migration assays. Exposure time to the various test agents varied: L-NAME (1 mM) 5 - 60 minutes; L-arginine (5 mM) 5 - 60 minutes after L-NAME pretreatment (60 minutes); ODQ (20 μM) 60 minutes; 8-Br cGMP (250 μM) 5 - 60 minutes; and PD098059 10 μM (30 minutes). After treatment, cells were rinsed twice with cold PBS and lysed with RIPA buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.5; 1% Triton X-100; 1% deoxycholate; 0.1% SDS; and 2 mM EDTA) containing phosphatase inhibitors (50 mM NaF and 1 mM Na_3VO_4, including a Complete Mini tablet; Boehringer Ingelheim GmbH, Mannheim, Germany). Total protein was determined using BCA protein assay reagent (Pierce Chemical Co., Brockville, ON, Canada). Ten percent polyacrylamide gels were loaded with 30 μg protein/well, and run at 100 volts for 1 hour; the proteins were transferred to PVDF membranes (100 volts; 1 hour; room temperature). The blots were then immunoprobed with mouse anti-phosphoERK primary (Santa Cruz; 1:1000 dilution in 5% milk) and goat anti-mouse IgG horseradish peroxidase conjugated secondary (Cedarlane Laboratories; 1:20,000 dilution in 5% milk) antibodies, and subsequently visualized using enhanced chemiluminescence (ECL Plus Western Blotting Detection System, Amersham Pharmacia Biotech, ON, Canada). For control purposed, blots were stripped, reprobed for total proteins, and subjected to chemiluminescence.

Data Analysis

Data from migration assays were analyzed using GraphPad PRISM® 3.0 (San Diego, CA), and treatment groups compared using one-way analysis of variance (ANOVA). A probability of 0.05 was used in determining statistical significance.

RESULTS

L-NAME and ODQ inhibit C3L5 cell migration in a dose-dependent manner

Figure 2 shows the migratory capacity of C3L5 cells treated with various concentrations of L-NAME (200 μM - 5 mM; 48 hour incubation) or ODQ (10 - 750 μM; 48 hour incubation). L-NAME treatment (i.e., 1.25 - 5 mM) reduced the migratory capacity of C3L5 cells relative to untreated control cells (Figure 1A), suggesting a role for NO in C3L5 cell migration. ODQ treatment (i.e., 50 - 750 μM) reduced migratory of C3L5 cells relative to untreated control cells (Figure 1B), suggesting a role for cGMP in C3L5 cell migration.

Stimulation of migration by endogenous NO is cGMP dependent

Figure 3 shows the migratory capacity of C3L5 cells under different treatment conditions (24 and 48 hour incubation). As previously demonstrated, the migratory capacity of C3L5 cells was reduced relative to control cells when treated with ODQ (50 uM: 24 and 48 hours, $P < 0.001$) or L-NAME (1 mM: 24 and 48 hours, $P < 0.01$). Migratory capacity of L-NAME-treated cells, additionally treated with L-arginine (5 mM), increased above basal levels (24 hours, $P < 0.05$; 48 hours, $P < 0.001$), suggesting a requirement for endogenous NO in C3L5 cell migration, and confirming that the effects of L-NAME are NO-specific. Presence of ODQ (i.e., L-NAME, L-Arginine and ODQ-treated cells) blocked the migration-restoring effects of L-arginine on L-NAME-treated cells (24 and 48 hours, $P < 0.001$), suggesting that endogenous NO-mediated stimulation of C3L5 cell migration requires a functioning MAPK pathway.

8-Bromo cGMP stimulates C3L5 cell migration

Figure 4 shows the migratory capacity of C3L5 cells under different treatment conditions (24 hour incubation). As previously demonstrated, the migratory capacity of C3L5 cells was reduced relative to control cells when exposed to ODQ ($P < 0.001$), and the combined treatment of L-NAME, L-arginine and ODQ ($P < 0.001$). The migratory capacity of C3L5 cells treated with 8-Br cGMP increased relative to control levels ($P < 0.05$), suggesting that elevation of intracellular cGMP stimulates migration of C3L5 cells.

Stimulation of migration by endogenous NO is MAPKK dependent

Figure 5 shows the migratory capacity of C3L5 under different treatment conditions (24 hour incubation). As previously demonstrated: 1) migratory capacity of C3L5 cells was reduced relative to control levels when treated with L-NAME ($P < 0.001$), and 2) migratory capacity of

L-NAME-treated cells additionally treated with L-arginine increased ($P < 0.01$), nearly reaching control levels ($P > 0.05$). The migratory capacity of C3L5 cells treated with PD098059 was reduced relative to control levels ($P < 0.001$) suggesting requirement of a functioning MAPK pathway for C3L5 cell migration. Treatment with PD098059 blocked the migration-restoring capacity of L-Arginine in L-NAME-treated cells, suggesting that endogenous NO-mediated stimulation of C3L5 cell migration requires a functioning MAPK pathway.

L-NAME reduces ERK1/2 phosphorylation

Figure 6 shows the effect of L-NAME (200 μM and 1 mM) on ERK1/2 phosphorylation as a function of time. For both concentrations, and all time points (i.e., 5 - 60 minutes), treatment of C3L5 cells with L-NAME reduced ERK phosphorylation relative to control cells, indicating that endogenous NO is required for basal ERK phosphorylation.

Endogenous and exogenous NO stimulate ERK1/2 phosphorylation

Figure 7A shows ERK1/2 phosphorylation in L-NAME-treated cells (60 minutes), additionally exposed to L-Arginine (NOS substrate; 5 - 60 minutes) or PD098059 (MEK inhibitor; 30 minutes). Additional exposure of L-NAME-treated cells to L-arginine increased ERK1/2 phosphorylation above control levels, suggesting that endogenous NO promotes ERK1/2 phosphorylation in C3L5 cells, and that the effects of L-NAME treatment on ERK1/2 phosphorylation are NO-specific. Treatment with PD098059 blocked ERK1/2 phosphorylation.
Figure 7B shows ERK1/2 phosphorylation in L-NAME-treated cells (60 minutes), additionally exposed to SNP (NO donor; 5 - 60 minutes) or PD098059 (30 minutes). Additional exposure of L-NAME-treated cells to SNP increased ERK1/2 phosphorylation above control levels, suggesting that exogenous NO promotes ERK1/2 phosphorylation in C3L5 cells, and that the effects of L-NAME-treatment on ERK1/2 phosphorylation are NO-specific. Treatment with PD098059 blocked ERK1/2 phosphorylation.

cGMP is required for ERK1/2 phosphorylation

Figure 8 shows the effect of L-NAME (200 μM, 60 minutes) ODQ (10 μM, 60 minutes) and 8-Bromo cGMP (250 μM, 5 - 60 minutes) on ERK1/2 phosphorylation in C3L5 cells. As previously demonstrated, treatment with L-NAME reduced ERK1/2 phosphorylation relative to untreated controls. ODQ reduced ERK1/2 phosphorylation relative to untreated controls, suggesting that cGMP is required for endogenous ERK1/2 phosphorylation. C3L5 cells treated

with 8-Br cGMP showed increased levels of ERK1/2 phosphorylation relative to untreated control cells, suggesting that elevation of cGMP stimulates endogenous ERK1/2 phosphorylation.

NO-mediated stimulation of ERK1/2 phosphorylation is cGMP-dependent

Figure 9 shows the effect of the combination treatment of L-NAME (1 mM, 60 minutes), ODQ (10 μM, 60 minutes) L-Arginine (5 mM, 5 - 60 minutes) on ERK1/2 phosphorylation in C3L5 cells. Presence of ODQ in the combination treatment (i.e., L-NAME, L-Arginine and ODQ-treated cells) blocked the phosphorylation-restoring effects of L-arginine on L-NAME-treated cells, suggesting that cGMP is required for endogenous NO-mediated stimulation of MAPK phosphorylation of C3L5 cells, providing a sequential link between the NOS, GC and MAPK pathways in mediating migration of C3L5 cells.

DISCUSSION

Using a murine mammary adenocarcinoma model, previous studies in our laboratory have shown that eNOS expression by tumour cells is positively associated with tumour growth and metastasis (Lala and Orucevic, 1998; Jadeski et al., 2000). Therapy of C3H/HeJ mice bearing a highly metastatic, eNOS-expressing, C3L5 mammary adenocarcinoma with NOS inhibitors N^G-methyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) reduced the growth and metastasis of this tumour (Orucevic and Lala, 1996a, 1996b; Lala and Orucevic, 1998), providing a causal link between NO and tumour progression. Furthermore, we have identified multiple mechanisms responsible for NO-mediated stimulation of tumour growth and metastasis in this tumour cell line. Tumour-derived NO promoted a) tumour cell invasiveness, by altering the balance between matrix metalloproteases and their inhibitors (Orucevic et al., 1999), b) migratory capacity of tumour cells (Jadeski et al., 2000), and c) angiogenesis (Jadeski and Lala, 1999).

Since capacity for migration is an essential component of cellular invasiveness and metastasis, we examined the molecular mechanisms of NO-mediated stimulation of C3L5 tumour cell migration, specifically, the intracellular pathways of signal transduction. Our results show that NO-mediated migratory responses of C3L5 mammary adenocarcinoma cells are dependent on activation of GC followed by activation of MAPK.

Many physiological functions of NO are mediated by GC activation; binding of NO to the heme group in GC stimulates conversion of GTP to cGMP, an important intracellular second messenger that mediates NO functions (Wink et al., 1998). Therefore, we were interested in the relationship between GC activation and cellular migration (Figure 1). The MAPK pathway, was also examined; this is an important signaling pathway, involving sequential phosphorylations, and is implicated in the regulation of a wide variety of cellular functions including cell proliferation, migration and transmission of oncogenic signals. A component of the MAPK pathway, ERK, activates myosin light chain kinase, which in turn activates the cellular motility apparatus, resulting in cell motility (Klempke et al., 1998). Therefore, we were interested in the relationship between ERK and cellular migration. By examining the effects of NO and cGMP on ERK phosphorylation, we hoped to elucidate a potential link between the two pathways.

NO-mediated promotion of C3L5 cell migration utilizes GC and MAPK pathways

Reports vary concerning the effect of NO on cellular motility. For example, factors promoting iNOS expression/NO production in endothelial cells (e.g., HGF, IL-1βα, TNFα, endothelin) also promote cellular migration (Bussolino, 1992; Rosen, 1993; Szekanecz, 1994; Radomski, 1993; Szabo, 1995; Noiri et al., 1997). HGF-induced expression of iNOS was determined to act as an essential switch, initiating cellular migration in epithelial cells (Noiri et al., 1996). Conversely, TGF-β inhibits iNOS expression and migration of endothelial cells and vascular smooth muscle cells (Koyama et al., 1990; Merwin et al., 1991). Together, these data suggest a role for NO in stimulating cellular migration. However, opposite results have also been reported. For example, exogenously added NO donors were found to inhibit endothelial, vascular smooth muscle and hepatic stellate cell motility (Dubey et al., 1995; Lau and Ma, 1996; Sarkar et al., 1996; Railli et al., 2000). Migratory capacity of eNOS-transfected aortic smooth muscle cells was reduced relative to control cells, an effect attributed to NO-mediated decreases in matrix metalloproteases (Gurjar et al., 1999). These conflicting data may be explained by presence of confounding variables factors, with potential effects on cellular migration, such as NO concentration, source (i.e., iNOS versus eNOS) and exposure time.

In the present study, exposure of C3L5 mammary adenocarinoma cells to the NOS inhibitor, L-NAME, inhibited *in vitro* migration of C3L5 mammary tumour cells in a dose-dependent manner, suggesting, and confirming earlier findings, that endogenous NO (product of eNOS expression) promoted tumour cell migration (Jadeski et al., 2000). The migratory capacity of L-NAME-treated cells additionally exposed to the NOS substrate, L-arginine, was restored to control levels, providing evidence that C3L5 cellular migration requires endogenous NO, and that the migration-inhibitory effects of L-NAME were NO-specific.

Role of cGMP in cellular migration has generally been reported as inhibitory, in the case of vascular smooth muscle cells (SMCs). For example, basal cGMP production induced by iNOS-derived NO inhibited migration of vascular SMCs (Kahn et al., 2000). Coinfection of cultured rat aortic SMCs with Adα1 and Adβ1 (GC α_1 and β_1 subunits) increased NO-stimulated intracellular cGMP levels and decreased migration (Sinnaeve et al., 2001). NO and cGMP were reported to decrease phosphotyrosine levels of focal adhesion proteins in primary cultures of aortic SMCs via activation of protein tyrosine phosphatases (PTPs) (Dhaunsi et al., 1997; Kaur et al., 1998). NO was later found to increase PTP-1B, a factor required for NO-induced inhibition of cell motility (Hassid et al., 1999). In contrast, our investigation of mammary tumour cells revealed a strong positive association between GC activation and

migratory capacity. To investigate the relationship between cGMP and migration, C3L5 cells were treated with ODQ (selective GC inhibitor) or 8-Br cGMP (cGMP analogue). Treatment with ODQ reduced, and 8-Br cGMP increased, migratory capacity of C3L5 cells relative to untreated controls. These data suggest that GC is required for C3L5 migratory function. Furthermore, presence of ODQ blocked the migration-restoring effects of L-Arginine on L-NAME treated cells, suggesting that cGMP is required for NO-mediated migratory responses of C3L5 cells.

Evidence exists, suggesting that MAPK signaling regulates cell motility. Cell migration involves myosin light chain kinase (MLCK) phosphorylation leading to actin-myosin association and cell contraction (Klempke et al., 1998). In COS-7 cells, ERK was found to phosphorylate MLCK, leading to increased myosin light chain phosphorylation and assembly of actin-myosin motors, an event necessary for cell contraction (Cheresh et al., 1999). A functioning MAPK pathway was required for TGF-β_1-induced migration of keratinocytes; treatment of keratinocytes with PD098059 (MEK inhibitor) impaired basal, and TGF-β_1-stimulated cell motility (Santibanez et al., 2000). The present study corroborates these findings; treatment with the MAPKK (MEK) inhibitor PD098059 reduced the migratory capacity of C3L5 tumour cells relative to untreated control cells, suggesting that a functioning MAPK pathway is required for C3L5 migration.

C3L5 cell migration depends on NO-stimulation of ERK1/2 phosphorylation

NO has been shown to have conflicting roles on the MAP kinase activation induced by various stimuli. For example, NO has partial or no effect on MAP kinase activation in certain cells (Chiu et al., 1999; Ingram et al., 2000; Mitani et al., 2000), whereas it stimulates MAPK activation in other cell types (Callsen et al., 1998; Komalavilas et al., 1999; Lander et al., 1996; Parenti et al., 1998).

C3L5 cells treated with L-NAME demonstrated reduced ERK phosphorylation relative to controls, indicating a relationship between endogenous NO and ERK phosphorylation. Additional treatment of L-NAME-treated cells with either L-arginine or SNP increased ERK phosphorylation above control levels, suggesting that endogenous and exogenous NO promotes MAPK phosphorylation in C3L5 cells, and that the effects of L-NA ME treatment on ERK phosphorylation are indeed NO-specific.

NO-mediated ERK phosphorylation of C3L5 cells is GC-dependent

A number of studies have investigated intermediary signaling elements of NO in promoting MAPK activation; conflicting results have been reported. Rat mesangial cells exposed to NO-liberating agents (e.g., S-nitrosoglutathione, 3-morpholinosynonimine) exhibited an early and late increase in MAPK (p42/p44) activation; rapid activation was cGMP-mediated, late activation was cGMP-independent (Callsen et al., 1998). Exposure of rat adventitial fibroblasts to SNP increased ERK1/2 activity; this effect was inhibited by ODQ and mimicked by 8-Br cGMP, implicating cGMP in the NO-mediated MAPK pathway (Gu et al., 2000). Likewise, ODQ blocked NO-mediated activation of ERK1/2 in endothelial cells, suggesting that NO and cGMP contribute to VEGF-dependent ERK1/2 activation (Parenti et al., 1998). In contrast, NO was found to inhibit stretch-induced increases in MAPK activity; 8-Br cGMP reduced strain-induced ERK activity, suggesting that the effect of NO on reducing MAPK activity was through cGMP generation (Ingram et al., 2000*a*; 2000*b*). In the present study, treatment with ODQ reduced, and 8-Br cGMP increased, ERK phosphorylation of C3L5 cells relative to untreated controls, suggesting that cGMP is required for ERK phosphorylation. Furthermore, PD098059 blocked the migration-restoring effect of L-arginine on L-NAME-treated cells, suggesting that endogenous NO-mediated stimulation of C3L5 cell migration requires a functioning MAPK pathway. Finally, presence of ODQ blocked the ERK phosphorylation-restoring effects of L-arginine on L-NAME-treated cells, suggesting that cGMP is required for endogenous NO-mediated MAPK phosphorylation, and providing a sequential link between the NOS, GC and MAPK pathways in mediating migration of C3L5 cells.

To our knowledge, the present findings constitute the first report of signal transduction mechanisms underlying NO-stimulation of migration in cancer cells. Since the C3H/HeJ mammary tumour model used in the present study shares many similarities with human breast cancer, including stimulation of tumour progression and metastasis resulting from endogenous NO (Thomsen et al., 1995; 1998), and migration is an essential step in invasion and metastasis, this study is relevant to designing new therapeutic approaches in breast cancer treatment.

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FIGURE LEGENDS

Figure 1. Molecular mechanisms of NO-mediated stimulation of C3L5 tumour cell migration examined in the present study. The NO metabolic pathway: NO is produced from L-arginine, by action of NOS. In tum, NO stimulates GC, mediating production of intracellular mediator cGMP. MAPK pathway: MAPK (ERK) activates myosin light chain kinase, which activates cellular motility apparatus, resulting in cell motility. The roles of the GC and MAPK pathways in NO-mediated effects on C3L5 cell migration was examined, and a potential link between them elucidated using a variety of agents, either alone or in combination, to stimlate or inhibit components of the pathways.

Figure 2. Migratory capacity of C3L5 cells treated with various concentrations of L-NAME (200 μM - 5 mM; 48 hour incubation) and ODQ (10 - 750 μM; 48 hour incubation). The migratory capacity of C3L5 cells was reduced in a dose dependent manner relative to control cells when treated either L-NAME ($P < 0.001$) or ODQ ($P < 0.001$).

Figure 3. Migratory capacity of C3L5 cells under different treatment conditions (24 and 48 hour incubation). The migratory capacity of C3L5 cells was reduced relative to control cells when treated with ODQ (50 μM; $P < 0.001$) or L-NAME (1 mM; $P < 0.01$). Migratory capacity of L-NAME-treated cells (1mM), additionally treated with L-arginine (5 mM), increased above basal levels (24 hours, $P < 0.05$; 48 hours, $P < 0.001$). Additional exposure of cells to ODQ [i.e., L-NAME (1 mM), L-arginine (5 mM) and ODQ (20 μM)-treated cells] inhibited the migration-stimulating capacity of L-arginine.

Figure 4. Migratory capacity of C3L5 cells under different treatment conditions (24 hour incubation). The migratory capacity of C3L5 cells was reduced relative to control cells when exposed to ODQ (20 μM; $P < 0.001$), and the combination of L-NAME (1mM), L-arginine (5 mM) and ODQ (20 μM) ($P < 0.001$). The migratory capacity of C3L5 cells treated with 8-Br cGMP (250 μM) was increased relative to control levels ($P < 0.05$).

Figure 5. Migratory capacity of C3L5 cells under different treatment conditions (24 hour incubation). The migratory capacity of C3L5 cells was reduced relative to control levels when treated with L-NAME (1 mM) ($P < 0.001$). Migratory capacity of L-NAME-treated cells (1mM) additionally treated with L-arginine (5 mM) increased ($P < 0.01$), nearly reaching control levels ($P > 0.05$). The migratory capacity of C3L5 cells treated with PD098059 (10 μM) was reduced relative to control cells ($P < 0.001$), and this reduction in migratory capacity was not reversed with L-arginine treatment (5 mM).

Figure 6. Effect of L-NAME on MAPK (ERK 1/2) phosphorylation. Treatment of C3L5 cells with various concentrations of L-NAME (i.e., 200 μM and 1 mM) reduced ERK 1/2 phosphorylation at all time points (i.e., 5 - 60 minutes) relative to control cells.

Figure 7. Phosphorylation of MAPK (ERK 1/2) under different treatment conditions.
Figure 7A: MAPK phosphorylation of untreated C3L5 cells, and L-NAME-pretreated C3L5 cells (1mM) that were additionally exposed to L-arginine (5 mM; 5 - 60 minutes), or PD098059 (10 μM). Additional treatment with L-arginine increased phosphorylation above control levels, and treatment of cells with PD098059 blocked phosphorylation.
Figure 7B: MAPK phosphorylation of untreated C3L5 cells, and L-NAME-pretreated C3L5 cells (1 mM) that were additionally exposed to SNP (300 μM; 5 - 60 minutes) or PD098059 (10 μM). Additional treatment with SNP increased phosphorylation above control levels, and treatment of cells with PD098059 blocked phosphorylation.

Figure 8. Phosphorylation of MAPK (ERK 1/2) under different treatment conditions. Treatment of C3L5 cells with L-NAME (1mM) or ODQ (20 μM) reduced, and 8-Br cGMP (250 μM) increased, ERK 1/2 phosphorylation relative to control cells.

Figure 9. Effect of L-NAME, ODQ and L-arginine treatment on phosphorylation of MAPK (ERK 1/2). Combined treatment of C3L5 cells with L-NAME (1 mM), ODQ (20 μM) and L-arginine (5 mM) reduced ERK 1/2 phosphorylation relative to control cells.

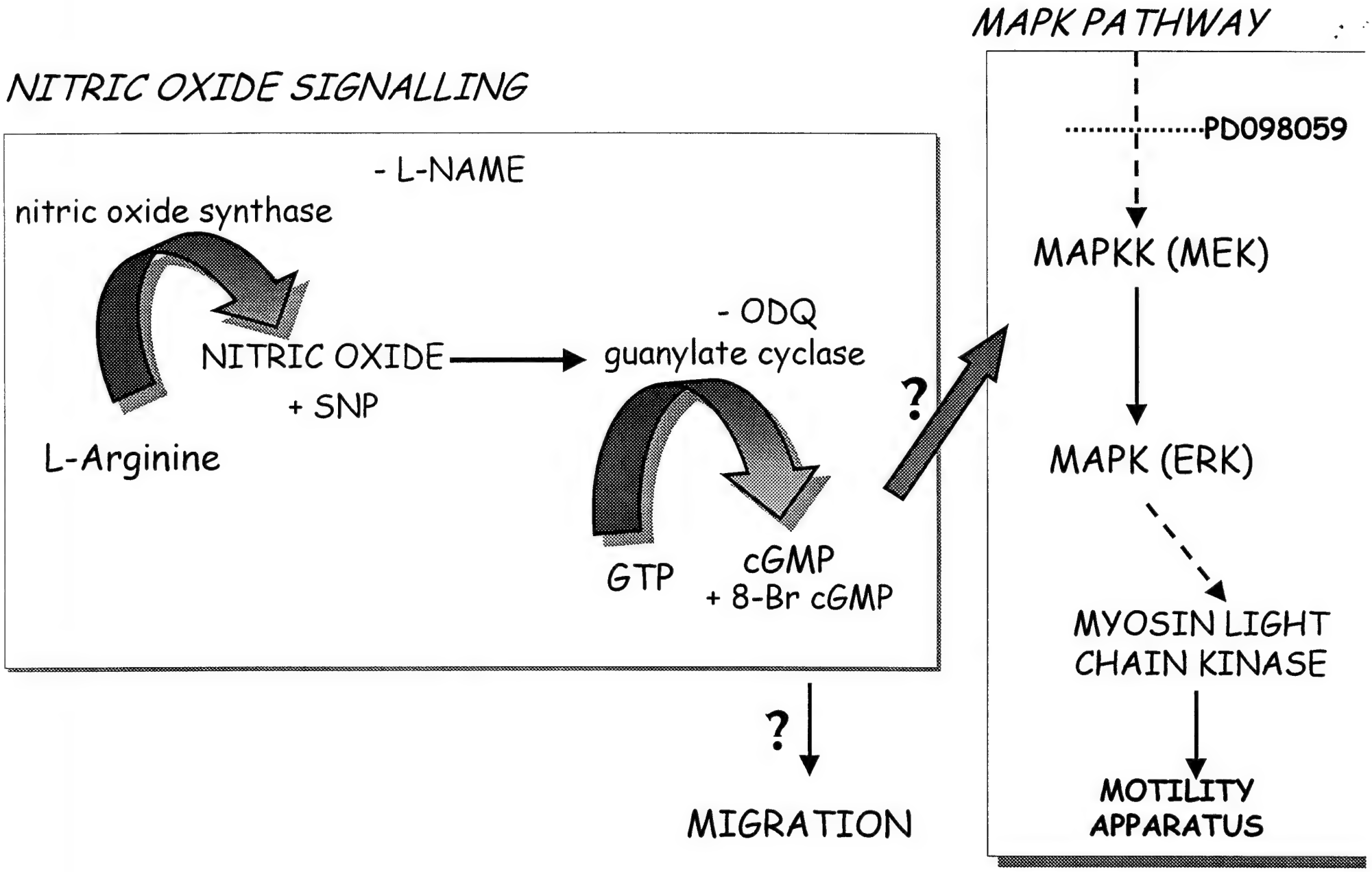
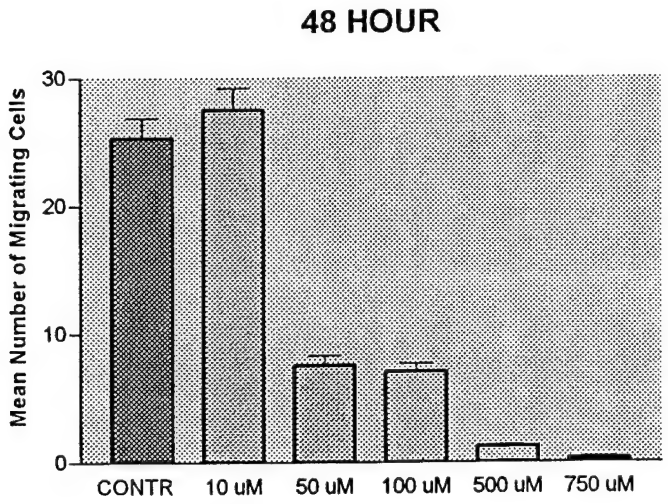

MAPK PATHWAY
NITRIC OXIDE SIGNALLING
PD098059
- L-NAME
nitric oxide synthase
MAPKK (MEK)
- ODQ
guanylate cyclase
NITRIC OXIDE
+ SNP
?
MAPK (ERK)
L-Arginine
GTP
cGMP
+ 8-Br cGMP
MYOSIN LIGHT
CHAIN KINASE
?
MOTILITY
APPARATUS
MIGRATION

FIGURE 1


48 HOUR
Mean Number of Migrating Cells
30
20
10
0
CONTR
10 uM
50 uM
100 uM
500 uM
750 uM

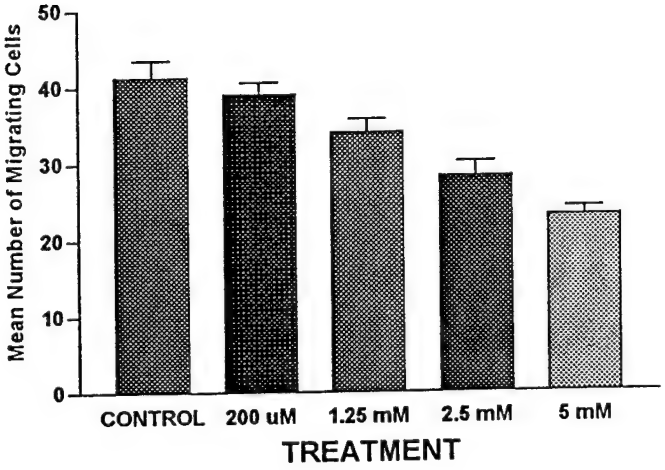
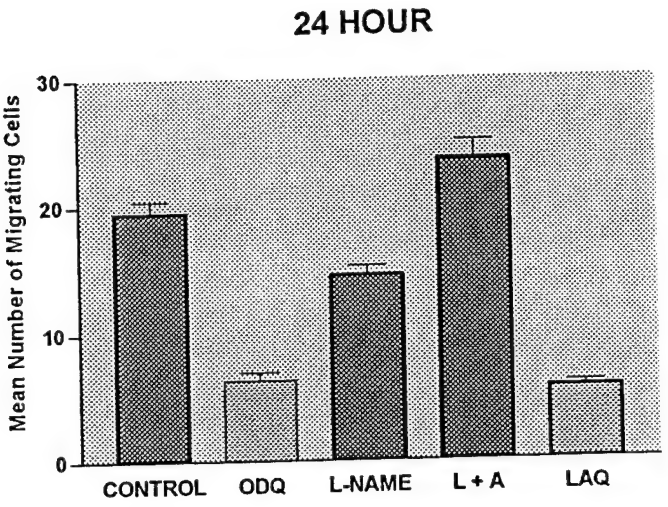

Mean Number of Migrating Cells
50
40
30
20
10
0
CONTROL
200 uM
1.25 mM
2.5 mM
5 mM
TREATMENT

FIGURE 2


24 HOUR
Mean Number of Migrating Cells
30
20
10
0
CONTROL
ODQ
L-NAME
L + A
LAQ

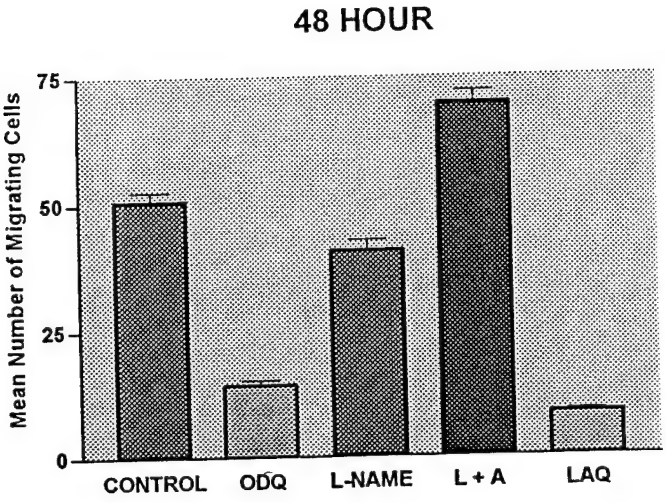

48 HOUR
Mean Number of Migrating Cells
75
50
25
0
CONTROL
ODQ
L-NAME
L + A
LAQ

FIGURE 3

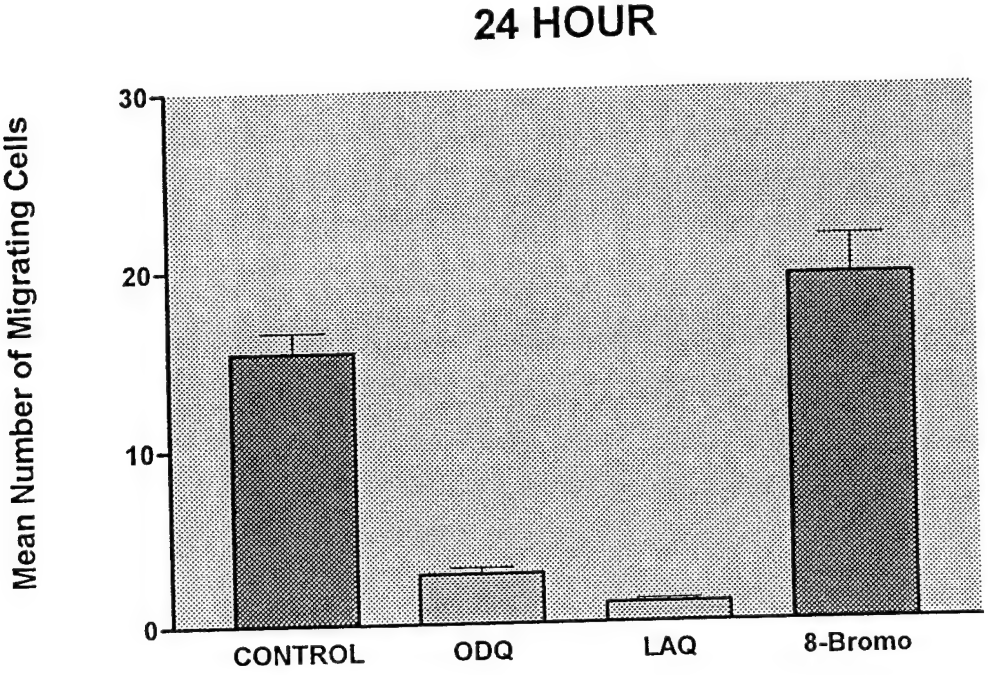

24 HOUR
Mean Number of Migrating Cells
30
20
10
0
CONTROL
ODQ
LAQ
8-Bromo

FIGURE 4

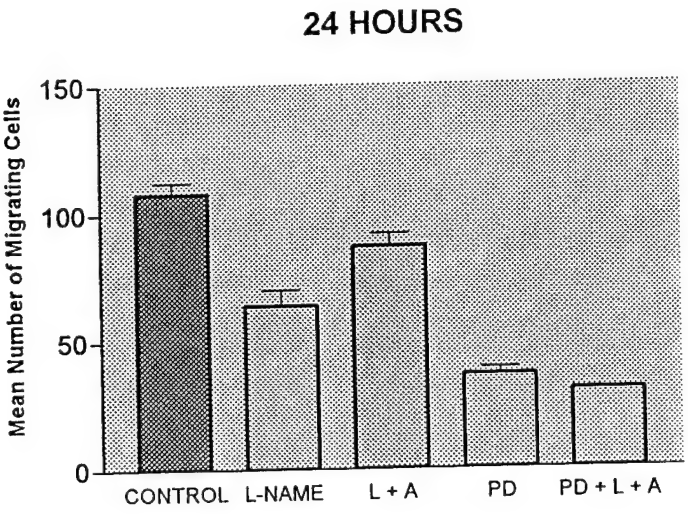

24 HOURS
Mean Number of Migrating Cells
150
100
50
0
CONTROL
L-NAME
L + A
PD
PD + L + A

FIGURE 5

Effect of L-NAME on ERK1/2 Phosphorylation

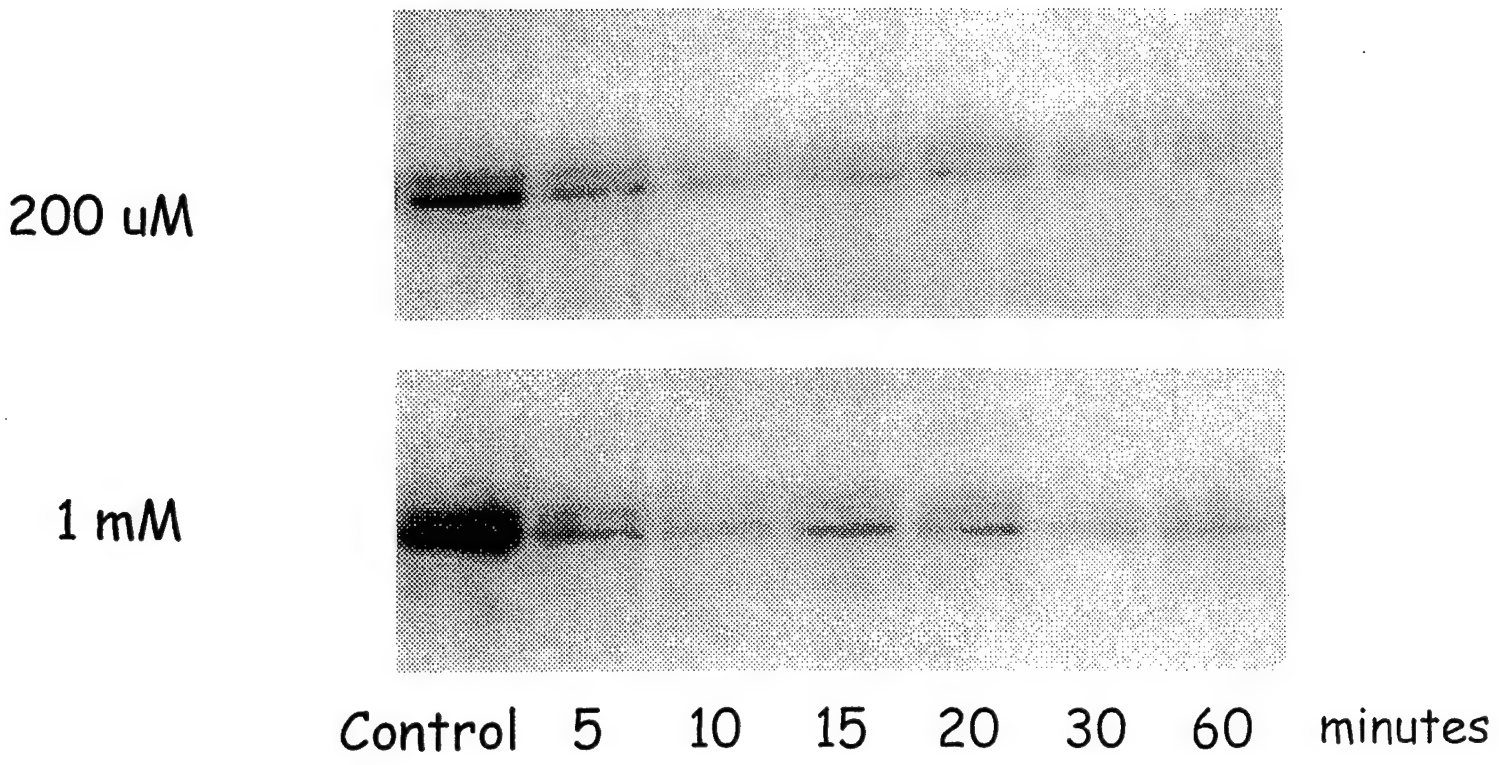


FIGURE 6

ENDOGENOUS *AND* EXOGENOUS NO STIMULATE ERK1/2 PHOSPHORYLATION

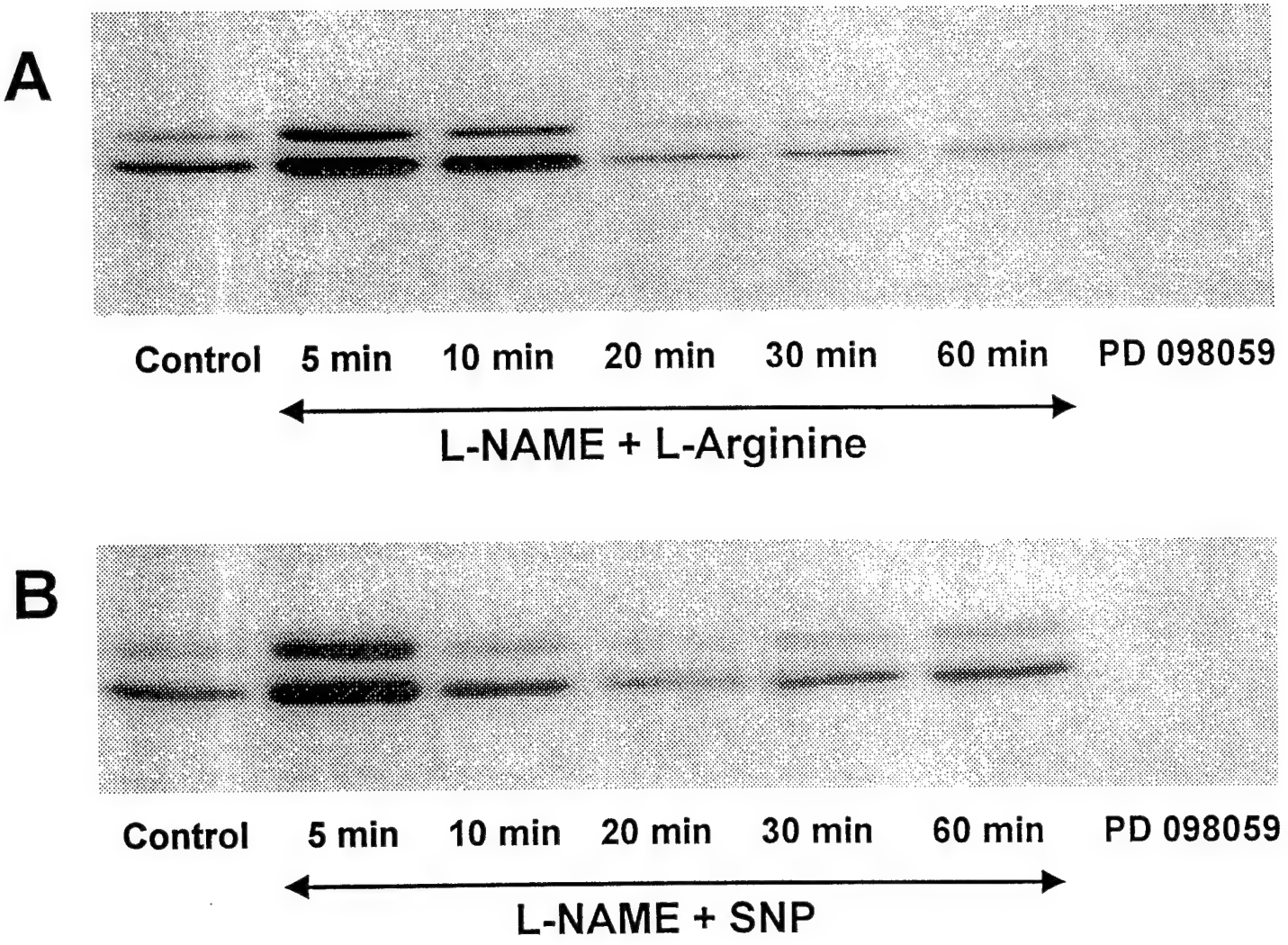


FIGURE 7

RELATIONSHIP BETWEEN cGMP AND ERK1/2 PHOSPHORYLATION

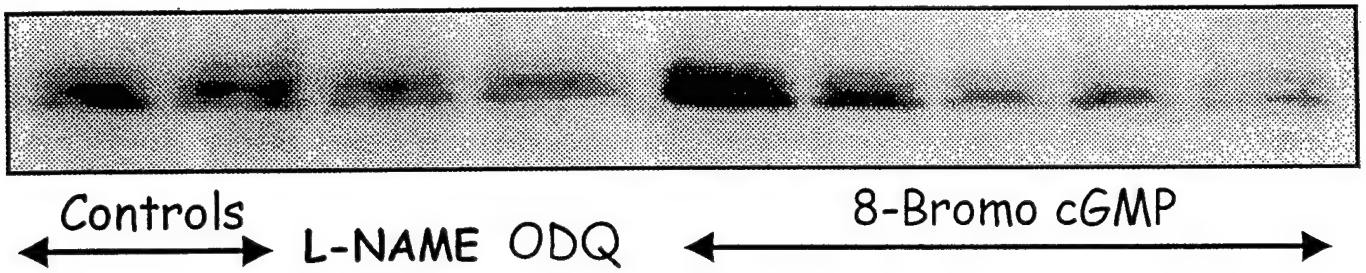


FIGURE 8

IS NO-MEDIATED STIMULATION OF ERK1/2 PHOSPHORYLATION cGMP-DEPENDENT?

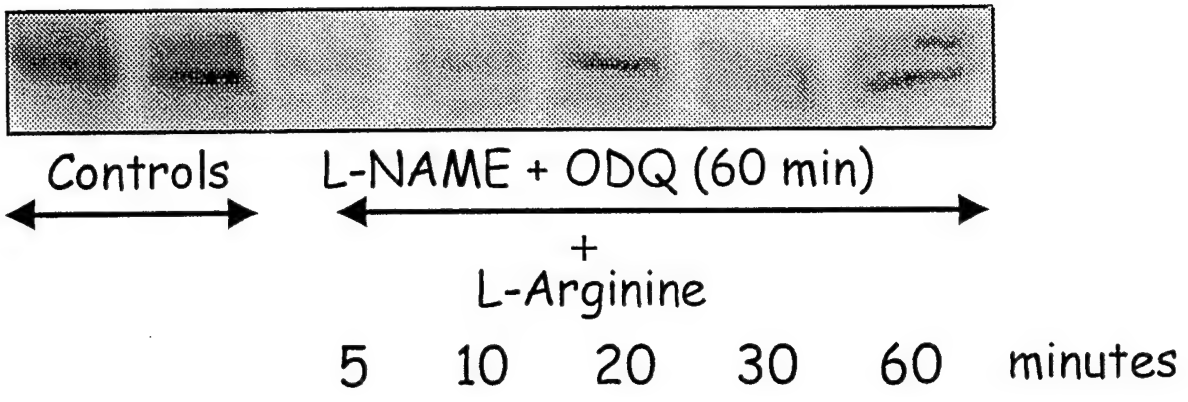


FIGURE 9

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Y. Lan[a],

Immunotherapy of C3H/HeJ mammary adenocarcinoma with interleukin-2, mistletoe lectin or their combination: effects on tumour growth, capillary leakage and nitric oxide (NO) production

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Abstract

Clinical application of interleukin (IL)-2-based immunotherapy of cancer has been limited by a major side-effect known as 'capillary leak syndrome', resulting from nitric oxide (NO) overproduction. A galactoside-specific lectin from *Viscum album* L. (VAA) has been reported to induce certain lymphokines and upregulate IL-2 receptors on lymphocytes. Present study was, therefore, designed to compare the effects of combination therapy with IL-2 (10^4 Cetus units/mouse, intraperitoneal (i.p). every 8 h, given as 5 day rounds per week, for one or two rounds) and VAA (1 ng/kg subcutaneous (s.c.), biweekly) with those of IL-2 or VAA therapy alone in C3H/HeJ female mice bearing s.c. transplants of a highly metastatic C3L5 mammary adenocarcinoma. IL-2 therapy alone reduced tumour growth and metastasis, but caused significant water retention indicative of capillary leakage in the kidneys after both rounds of therapy, whereas pleural effusion was only evident after the first round and not the second round. A sharp rise in the systemic NO levels after the first round, followed by a decline after the second round of IL-2 therapy suggested a causal relationship of increased NO levels to pleural effusion. A strong immunostaining for nitrotyrosine (a marker for the production of peroxynitrite) was noted in the renal tubules at the end of both rounds of therapy suggestive of a causal association of this toxic NO-metabolite with capillary leakage in the kidneys. Addition of VAA to IL-2 therapy had no effect on any of the above parameters. Unexpectedly, however, VAA therapy alone stimulated tumour growth as well as lung metastases. NO induction in the C3L5 cells by VAA was excluded as a possible reason for this stimulation. Present results suggest the need for exercising caution in the use of VAA as an immunoadjuvant in human cancer therapy.

Keywords: Alternative (complementary) medicine; Immunotherapy; Interleukin-2; Lectin; Mistletoe; Nitric oxide; Biological response modifier

1. Introduction

High dose interleukin (IL)-2 therapy has shown efficacy in animal [1–4] as well as human [5–7] cancer models. However, its application has been limited by a life-threatening side-effect called capillary leak syndrome which is characterised by a rapid fluid accumulation in the tissue spaces and severe hypotension [8]. Combination of chronic indomethacin therapy with IL-2 therapy was shown to improve antitumour and antimetastatic effects of IL-2 therapy because of the improved activation of lymphokine-activated killer (LAK) cells *in situ* [2,3], but have no effect on IL-2-induced capillary leakage [9]. We subsequently discovered that endothelial damage responsible for this leakage resulted primarily from an overproduction of nitric oxide (NO) due to iNOS induction *in vivo* and that IL-2-induced capillary leakage can be ameliorated by combined therapy with NOS inhibitors [10–12]. A possible beneficial role of other biological response modifiers in improving the therapeutic efficacy and reducing the toxicity of IL-2 therapy has not been explored.

In recent years, immunostimulating effects of certain lectins have evoked considerable interest for application in cancer biotherapy. The galactoside-specific lectin from *Viscum album* L. (VAA or formerly ML-1) is considered to be the key immunostimulating component of

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commercially available mistletoe extracts historically employed in phytotherapy [13–14]. *In vitro*, VAA has been reported to upregulate the gene expression and secretion of proinflammatory cytokines including IL-1-α, IL-1-β, IL-6, IL-12, tumour necrosis factor (TNF)-α, interferon (IFN)-γ, and granulocyte macrophage-colony stimulating factor (GM-CSF) by human leucocytes [15–18]. Furthermore, VAA was shown to stimulate natural killer (NK) cell activity in rat splenocytes [18], as well as NK and LAK cell cytotoxicity in cultures of human peripheral blood lymphocytes [19]. Administration of VAA by parenteral routes was shown to stimulate the activity and incidence of NK cells in healthy rabbits, rats and human breast cancer patients [18,20] and upregulate the expression of IL-2 receptors (CD25) on lymphoid cells of breast cancer patients [21]. The therapeutic value of the administration of VAA and VAA-containing extracts remains a highly debated area because of reports indicating beneficial, as well as detrimental effects. VAA therapy was shown to reduce experimental metastasis formation by murine sarcoma [22] lymphoma [23] and melanoma [24] cells, reduce the growth of a rat glioma [25], and improve the survival of immunodeficient mice transplanted with a human ovarian carcinoma cell line [26]. However, VAA therapy stimulated the growth of chemically-induced rat bladder carcinomas [27,28]. Application of mistletoe therapy to human cancer has been confounded with the problems of inadequate rationale and therapeutic designs and the questionable anticancer potential of lectin-induced cytokines which can stimulate as well as inhibit tumour growth [13,29,30]. VAA-induced stimulation of the proliferation of human tumour cells in histotypic cultures [31], and apparent stimulation of tumour growth at the injection site in a patient with non-Hodgkin's lymphoma [32] have raised additional concerns against VAA therapy.

In view of the putative immunostimulating function of VAA, including the ability to upregulate IL-2 receptors on lymphocytes, we wanted to test whether inclusion of non-toxic, but immunostimulant, doses of VAA could improve the antitumour and antimetastatic effects of IL-2 therapy without an adverse influence on the IL-2, induced capillary leakage. The present study was, therefore, designed to compare the effects of the combined therapy with IL-2 and VAA with those of IL-2 and VAA therapy alone in a highly metastatic C3L5 mammary adenocarcinoma model in C3H/HeJ mice. The following parameters were measured: tumour growth and spontaneous metastases in the lungs, capillary leakage (pleural effusion and wet/dry weight of the lungs and the kidneys), NO production (nitrite + nitrate levels in the serum, pleural fluids, kidney and lung homogenates), and the level of immunostaining for nitrotyrosine, which is a marker for the production of peroxynitrite, a toxic NO-metabolite implicated in capillary leakage.

2. Materials and methods

2.1. Reagents

Biotinylated anti-rabbit IgG and Vectastain ABC kit (PK-4000) were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Immunoaffinity-purified polyclonal IgG antibody to nitrotyrosine produced in rabbits were from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Lipopolysaccharide from *Escherichia coli* 026:B6 (LPS) and 3-nitro-L-tyrosine (antigen to neutralise nitrotyrosine antibody) were obtained from Sigma (Oakville, Canada). Nitrate/Nitrite Colorimetric Assay Kit was from Cayman Chemical (Ann Arbor, MI, USA). IFN-γ and all reagents to maintain cell culture were from GIBCO BRL (Burlington, Canada). Recombinant human IL-2 (Lot PA0522A) with activity of 18×10^6 International Units (World Health Organization (WHO)) or 3×10^6 Cetus Units/mg protein was kindly provided by the Chiron Corporation (Emeryville, CA, USA). Biochemically-pure and endotoxin-free VAA and its carbohydrate-binding B-chain were isolated from buffer extracts of *Viscum album* L. using lactose-Sepharose 4B as an affinity matrix in the chromatography [33].

2.2. Mice

C3H/HeJ female mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and were acclimatised in the animal quarters of the University of Western Ontario for 3 weeks before starting experiments. Mice were randomly separated into five groups, each of 8–10 animals/plastic cage, and were kept on 12-h light/dark cycle with free access to standard mouse chow and water *ad libitum*. The mean weight of the mouse was 22 g at the onset of experiments. The guidelines set by the Canadian Council of Animal Care were strictly followed during all treatments of mice.

2.3. Tumour cell line, murine tumour transplantation and measurement of tumour growth

C3L5 murine mammary adenocarcinoma cell line maintained in this laboratory is an extensively characterized clonal derivative of a spontaneous C3H/HeJ mammary tumour [3]. It is strongly positive for endothelial (e) NOS, and NO production by the tumour cells has been shown to promote tumour progression by stimulating tumour cell migration, invasiveness [34] and angiogenesis [34,35]. These cells can also be induced in the present of IFN-γ and LPS to produce additional NO which stimulates their invasive function *in vitro* [36]. The cells were grown in monolayer in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented

with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified incubator fed with 5% CO_2 at 37°C. Cells were harvested by brief exposure to 0.05% trypsin-phosphate-buffered solution-ethylene diamine tetra acetic acid (PBS-EDTA) and resuspended in RPMI-1640 medium at a concentration of 10^6 cells/ml. The cell viability was more than 96% according to a dye exclusion test with trypan blue. Tumour cell transplantation was done by subcutaneous (s.c.) injections of 5×10^5 viable cells in 0.5 ml cell suspension in the mammary line of the left axillary region of C3H/HeJ female mice. Starting at 1 week after tumour transplantation, the minimal and maximal diameters (a and b) of the tumours were measured on alternate days using digital calipers and the tumour volume was calculated according to the equation $V=0.52a^2b$ [34].

2.4. Experimental protocol for treatment of mice with IL-2 and VAA

Two series of experiments were conducted utilising a total of 90 mice. In the first series, five experimental groups of mice ($n=10$ animals/group) were employed, namely (1) healthy mice, (2) control tumour-bearing mice, (3) IL-2- treated tumour-bearing mice, (4) VAA-treated tumour-bearing mice, (5) combined IL-2+VAA-treated tumour-bearing mice. The treatment of mice with both drugs was started on day 7 after tumour transplantation. The groups (3) and (5) received two rounds of therapy with IL-2 as intraperitoneal (i.p.) injections at a dose of 10^4 Cetus Units (i.e. 6×10^4 IU)/mouse every 8 h during 5 days, followed by a 4-day recess and then a similar 5-day round with IL-2. IL-2 was dissolved in RPMI-1640 medium at a concentration of 10^6 Cetus Units/ml, so that each injection was 100 μl of the solution. The treatment of mice with VAA was in accordance with the dosage and schedule established for immunomodulation, namely a non-toxic dose 1 ng/kg body weight biweekly [13,20,37]. This protocol is also in line with clinical recommendations by commercial distributors of standardised mistletoe extracts in Germany. The drug preparation was deliberately designed to match earlier animal studies [27,28]. In brief, lyophilised VAA was freshly dissolved in PBS solution containing 50 μg/ml of mouse albumin (MA) to prevent lectin adsorption on the tube surface, diluted up to a concentration of 0.22 ng/ml in the same vehicle and injected s.c. with 100 μl of the solution on days 7, 10, 13, 16, and 19 after tumour transplantation. Mice in control groups (1) and (2) received blank injections of RPMI-1640 and PBS-MA. On day 21 after tumour transplantation, and 2 h after the last injection of IL-2, mice were killed by i.p. injection of 100 μl of euthanol solution (135 mg/ml) and samples of blood were collected and the lungs and kidneys were removed for further analyses.

In a second series of experiments, five similar groups of animals were used as above except for the fact that 8 animals were used per group and the animals were killed at the end of one round of therapy (on day 5 after the initiation of therapy, that is, 12 days after tumour transplantation).

2.5. Measurements of lung metastases

Right lungs were inflated with Bouin's fixative and the visible metastatic colonies which stand out on the lung surface were counted under a dissecting microscope. Metastasis count was only possible in the first experimental series representing animals at 21 days following tumour transplantation, i.e. those receiving two rounds of therapy. No visible lung metastasis appeared in any of the animals in the second series, in which case animals were killed at 12 days after tumour transplantation.

2.6. Measurements of capillary leakage

Fluid accumulation in the pleural cavity is a major sign of the capillary leak syndrome. The volume of pleural effusion, if any, was measured by removal from the pleural cavities as reported earlier [12] before removing the lungs. Increased water retention in the organs is a good measure of capillary leakage. Left lungs and kidneys were weighed (wet weight) immediately after removing and placed into a freezer at −20°C. Shortly thereafter, the collected samples were freeze-dried for 48 h to a constant weight. The weight of the dried samples was measured (dry weight) and the wet/dry weight ratios of organs were calculated as an indicator of the water content in these organs to assess capillary leakage [10,11].

2.7. Measurements of NO in blood serum and tissue homogenates

The coagulated blood was centrifuged for 10 min at 15000 rpm using the Biofuge 17 R (Baxter Scientific Products), the serum was collected into Eppendorf tubes and kept at −20°C. To analyse nitrite and nitrate levels in the tissues, the freeze-dried left lungs and kidneys were used. Each tissue sample was homogenised in 15 or 40 volumes of PBS, pH 7.35, depending on the initial wet weight of the lung or kidney, respectively, using Polytron PT-MR2100 from Kinematica AG, CH. The homogenates were centrifuged at 4°C for 30 min at 15000 rpm and the supernatants were collected into Eppendorf tubes, which were kept on ice. Both blood serum and tissue homogenates were ultrafiltered through 10 kDa molecular weight cut-off Ultrafree filters from regenerated cellulose membrane (Sigma) to get protein-free samples prior to NO analyses. Nitrate/Nitrite

Colorimetric Assay Kit from Cayman Chemical (Ann Arbor, MI, USA) was applied to measure total nitrate + nitrite ($[NO_2^- + NO_3^-]$) concentration in deproteinised serum and supernatants of the tissue homogenates. This assay is based on the conversion of nitrate to nitrite by nitrate reductase followed by application of the Griess reagent. In the case of lung and kidney homogenates, the water content of these organs and the dilution factor in making homogenates were applied in computing the $[NO_2^- + NO_3^-]$ levels.

2.8. Study of lectin effects on NO synthesis by C3L5 cells in vitro

To measure NO synthesis by C3L5 cells, the RPMI-1640 media was replaced by the Dulbecco's modified Eagle medium, which contains a negligible level of nitrate (2.5×10^{-7} M ferric nitrate). Aliquots (800 μl) of a cell suspension (2×10^5 cells/ml) were grown in 24-well plates in triplicate in standard conditions with or without the presence of VAA or its non-toxic carbohydrate-binding B-chain (1, 5, 10, 50, 100, 1000 ng/ml) as well as LPS (10 μg/ml) + IFN-γ (500 U/ml). The culture media were collected at 48 h and processed with the Cayman kit to measure the $[NO_2^- + NO_3^-]$ concentration.

2.9. Immunostaining for nitrotyrosine

Nitrotyrosine is produced as a result of nitration of tyrosine residues of cellular proteins (including tyrosine kinases) by peroxynitrite which is a reaction product of NO with superoxide. Thus immunostaining for nitrotyrosine is considered to be a reliable marker for peroxynitrite production *in vivo* [38]. We have strong evidence to suggest that NO-mediated capillary leakage induced by IL-2 therapy is due to the formation of peroxynitrite and that nitrotyrosine immunostaining of the kidneys provides a good reflection of IL-2 therapy-induced peroxynitrite formation (Lala, data not shown). The right kidneys were removed from mice and fixed in 10% buffered neutral formalin (VWR Scientific Products, West Chester, PA, USA). The paraffin-embedded samples were cut to get 4 μm-thick sections, which were then routinely processed for immunohistochemical staining. Briefly, the deparaffinised sections were incubated for 10 min at room temperature with 3% H_2O_2 in methanol to inhibit endogenous peroxidases, blocked for 1 h with 10% normal goat serum in 1% bovine serum albumin (BSA)-PBS solution, treated overnight with the primary rabbit polyclonal anti-tyrosine antibody (1:500 dilution) at 4°C, and then for 45 min at room temperature with the secondary antibody (biotinylated anti-rabbit IgG, 1:500 dilution). The ABC-kit from Vector was then applied for 45 min at room temperature. Specificity controls were obtained by combined application of the primary antibody with 0.01 M 3-nitro-L-tyrosine which neutralises the antibody. The visualisation of nitrotyrosine sites in the kidney sections was done using 3,3′-diaminobenzidine as a peroxidase substrate. The immunostained sections were finally counterstained lightly with Mayer's haematoxylin, dehydrated and mounted with Permount (Fisher Scientific, Fair Cawn, NJ, USA). They were analysed under an axiophot (Zeiss) microscope and pictures were recorded at 630× magnification with a digital image recorder.

2.10. Statistics

Each group of mice at the time of final data analysis contained 7–10 animals because of death of an occasional animal in the IL-2-treated groups prior to data collection. The mean values, standard deviations, P values from the Student's test, and Pearson correlation coefficients were calculated using Microsoft® Excel tools. For analysing data on pulmonary metastases, the Mann–Whitney rank sum test was applied, because of non-parametric distribution of the data. P values of less than 0.05 were considered significant.

3. Results

3.1. Effects of therapies on subcutaneous tumour growth and pulmonary metastasis

Growth curves for C3L5 tumours in the various treated groups are presented in Fig. 1. Administration of IL-2 (10^4 Cetus Units/mouse, every 8 h) during two

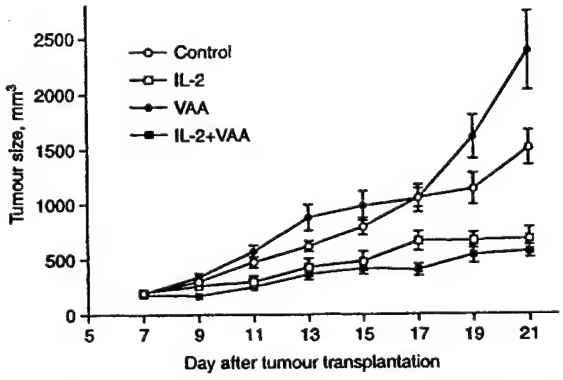

Fig. 1. Effects of intraperitoneal (i.p.) injection of interleukin-2 (IL-2) and subcutaneous (s.c.) injections of VAA, as well as their combination on tumour growth after s.c. inoculation of 5×10^5 C3L5 cells in C3H/HeJ female mice. Data indicate mean ± standard error (S.E.). First 3 data points (days 7, 9 and 11) are derived from both experimental series ($n=18$), whereas subsequent data points are derived from experimental series I ($n=9$–10), following the sacrifice of those in experimental series II on day 12. Significant ($P<0.05$) reduction of tumour growth was noted in both IL-2 and IL-2 + VAA-treated mice from day 13 onwards, whereas a stimulation ($P<0.05$) is noted in VAA-treated mice on day 19 or 20.

rounds of injections (days 7–11 and 16–21) significantly suppressed the tumour growth corroborating the previous data from this laboratory using different IL-2 treatment regimens and treatment periods [3,11]. However, the tumour growth was stimulated in the group of mice receiving biweekly s.c. injections of VAA alone at a dose of 1 ng/kg (Fig. 1) starting at day 7 after tumour cell transplantation. When VAA was combined with IL-2 therapy, the tumour growth was suppressed to the same extent as noted with the IL-2 therapy alone.

The rates of primary tumour growth were positively correlated with the number of lung metastases visible at 3 weeks after tumour transplantation in the various treated groups, as presented in Table 1. The incidence of lung metastasis was significantly increased by VAA therapy and reduced equally in both animal groups treated with IL-2 alone or combined IL-2 + VAA. Furthermore, the size of visible lung nodules was consistently much larger in the VAA-treated animals, compared with the other groups (data not shown). Histological examination of the lungs (data not shown) revealed more frequent and larger foci of intrapulmonary metastases in the VAA-treated group than in other groups confirming the macroscopic data.

3.2. IL-2-induced capillary leakage

The salient clinical feature of IL-2 therapy (but not VAA therapy)-induced capillary leakage in tumour-bearing mice was the development of pleural effusion during the first round of immunotherapy. The mean volumes of pleural effusions detected in mice after 5 days of IL-2 injections in the present protocol were 1.2±0.2 ml ($n=8$) and 1.1±0.2 ml ($n=8$) for IL-2 and IL-2 + VAA treated mice, respectively. These values were not significantly different from each other. Pleural effusion was the main cause of morbidity (six out of 36 mice in the two experimental series combined, receiving IL-2 or IL-2 + VAA, i.e. 17%) observed up to the end of the first round of immunotherapy. The volume of pleural fluid was more than 1.3 ml in these cases. No morbidity was noted in the other groups. Remarkably, mice surviving the first round of IL-2 therapy tolerated well the second round of IL-2 and no pleural effusion was observed in these animals at sacrifice. This finding confirms our earlier observations with a different IL-2 regimen [12].

An increase in the water content of organs (wet/dry weight ratio) is a good indicator of capillary leakage induced by IL-2 therapy [10,11]. Table 2 documents the data for the lungs and the kidneys after one and two rounds of therapy. Significant capillary leakage was identified in the lungs only in the IL-2 + VAA-treated group after one round of therapy, when water contents were compared with those in the control tumour-bearing mice. However, significant leakage was observed in the kidneys after both rounds of therapy in the IL-2- as well as IL-2 + VAA-treated groups. No difference in the water contents of the kidneys was observed between these two groups. Thus, in summary, the addition of VAA to IL-2 therapy neither reduced nor increased the extent of pleural effusion and fluid leakage in the kidneys.

Table 1
Effects of therapy with IL-2, VAA, and their combination on the number of visible metastatic colonies on the lung surface in mice sacrificed at 21 days after tumour transplantation in the experimental series I

Experimental group (number of mice)	Number of lung surface metastases	
	Range	Mean±S.D.
1. Control ($n=10$)	1–21	7.5±7.1
2. IL-2 treatment ($n=7$)	1–6	2.3±1.9*
3. VAA treatment ($n=10$)	5–61	27.6±22.3*
4. Combined IL-2 + VAA treatment ($n=9$)	0–9	2.3±2.8*

VAA, a galactoside-specific lectin from *Viscum album* L.; IL-2, interleukin 2; S.D., standard deviation.

*$P<0.028$ compared with the control group. Mice were killed on day 21 after subcutaneous (s.c.) transplantation of C3L5 cells (5×10^5 cells/mouse) into C3H/HeJ mice.

3.3. NO levels in the serum, pleural fluid and different tissues of IL-2- and VAA-treated mice

The level of $[NO_2^- + NO_3^-]$ in the serum, pleural fluid, kidneys and lungs of control and experimental mice was measured at two time points corresponding to the end of the first round of immunotherapy (day 12 after tumour transplantation) and the second round (day 21 after tumour transplantation). A significantly elevated level of $[NO_2^- + NO_3^-]$ ($P<0.03$) was detected in the blood serum, kidneys and lungs after the administration of IL-2 during the first round (Table 3). Additionally, a high level of $[NO_2^- + NO_3^-]$ was also observed in the pleural fluids of IL-2- and IL-2 + VAA-treated tumour-bearing mice, namely 152.2±59.5 μM ($n=8$) and 108.9±67.5 μM ($n=8$), respectively, very similar to the respective levels in the serum of the same animals. The correlation coefficients (Pearson) were 0.9824 and 0.9832 for the IL-2- and IL-2 + VAA-treated groups, respectively. Similarly, increased levels of $[NO_2^- + NO_3^-]$ were also observed in kidneys and lungs of IL-2- as well as IL-2 + VAA-treated mice (Table 3). In essence, $[NO_2^- + NO_3^-]$ levels in the various tissues appeared to reflect the common NO-status of the individual animal group, as defined by the activity of different NOS enzymes including those induced by IL-2. Furthermore, administration of VAA alone had no significant effect on the levels of $[NO_2^- + NO_3^-]$ in control or IL-2-treated mice (Table 3).

NO levels in the different groups after the second round of therapies are presented in Table 4. Unexpectedly, the levels of $[NO_2^- + NO_3^-]$ in all tested tissues were significantly reduced ($P < 0.03$) in IL-2 (±VAA)-treated animal groups when compared with control tumour-bearing or healthy mice. The reduced levels of $[NO_2^- + NO_3^-]$ correlated with the absence of any pleural exudations in these mice.

3.4. Nitrotyrosine expression in the kidneys

NO is a free radical which can rapidly combine with available oxygen free radicals. Peroxynitrite is such a reactive intermediate which may mediate the endothelial damage responsible for capillary leakage. This molecule is toxic, unstable and reacts with tyrosine residues in proteins to form stable 3-nitrotyrosine [38], which appears to be a reliable footprint of peroxynitrite formation. We had earlier established that normal lungs exhibit significant nitrotyrosine immunostaining because of high endogenous levels of NO in this organ (Lala, data not shown). However, kidneys were found to serve as a better indicator for IL-2-induced nitrotyrosine formation. The right kidneys of all the mice were processed for nitrotyrosine immunostaining. Kidney tissues from healthy and control tumour-bearing mice, as well as from VAA-treated mice demonstrated the absence of immunostaining or sparse, patchy, very light to light, immunostaining in both the cortex and medulla (Fig. 2a–d). However, IL-2 treatment of the tumour-bearing mice, alone or in combination with VAA, led to marked enhancement of immunostaining of the renal tubules in both the cortex and the medulla (Fig. 2, e and f). In addition, macrophages within the stroma (renal capsule) were strongly positive in these mice (data not shown).

The negative control samples (antibodies pretreated with 3-nitro-L-tyrosine) in all groups were devoid of immunostaining, indicating the specificity of the staining (Fig. 2g and h). Furthermore, no nitrotyrosine staining was seen in the glomeruli, adipose tissue (perirenal fat) and adrenal glands in treated as well as control groups, serving as intrinsic negative controls in these tissue sections. Remarkably, the strong expression of nitrotyrosine in the renal tubules was observed both after the first and the second rounds of IL-2 therapy. This was in contrast to the reduced level of $[NO_2^- + NO_3^-]$ in the different tissues including the kidney after the second round of IL-2 therapy, indicating that enhanced expression of nitrotyrosine persists in this organ probably resulting from the high NO levels during the first round of IL-2 therapy. These results reinforce the notion that the persistent peroxynitrite-mediated injury to the kidneys may be responsible for the capillary leakage, even in the absence of a high local NO level after the second round of IL-2 therapy.

Table 2
Water content in the left lung and kidney of treated mice, expressed as the wet/dry weight ratio of the organs (experimental series I and II)

Mice group	Wet/dry weight ratio, mean±S.D.			
	Lung		Kidney	
	1 round	2 rounds	1 round	2 rounds
1. Healthy mice	5.63±0.42 (8)	4.97±0.67 (10)	3.67±0.20 (8)	3.66±0.20 (10)
2. Control tumour-bearing mice	5.45±0.52 (8)	5.31±0.51 (10)	3.53±0.40 (8)	3.68±0.19 (10)
3. Treatment with IL-2	5.91±0.81 (8)	5.02±0.42 (7)	4.16±0.48 (8)**	4.07±0.15 (7)***
4. Treatment with VAA	5.63±0.58 (8)	4.94±0.44 (10)	3.63±0.21 (8)	3.86±0.29 (10)
5. Combined treatment with IL-2 and VAA	5.97±0.32 (8)*	5.05±0.60 (9)	4.23±0.35 (8)**	4.11±0.17 (9)***

IL-2, interleukin; VAA, a galactoside-specific lectin from *Viscum album* L.; S.D., standard deviation.
*$P < 0.02$, **$P < 0.01$, ***$P < 0.001$ compared with control tumour-bearing mice. Number in parentheses indicates the number of animals.

Table 3
The level of nitrate + nitrite (μM) in mice after one round of therapy (experimental series II)

Animal group	Blood serum	Pleural effusion	Left kidney	Left lung
1. Healthy mice	42.2±5.9 (8)		33.3±8.5 (8)	49.2±22.6 (8)
2. Control tumour-bearing mice	36.0±11.8 (8)		31.3±11.0 (8)	41.4±25.3 (8)
3. IL-2 treatment	145.6±51.9 (6)*	152.2±59.5 (8)	94.3±34.9 (8)***	160.2±59.1 (8)*****
4. VAA treatment	41.3±8.9 (8)		23.2±10.7 (8)	79.8±60.6 (8)
5. Combined IL-2 and VAA treatment	101.4±72.6 (7)**	108.9±67.5 (8)	69.8±35.9 (8)****	101.3±35.4 (8)******

IL-2, interleukin; VAA, a galactoside-specific lectin from *Viscum album* L.
*$P = 0.0016$, **$P = 0.0274$, ***$P = 0.000541$, ****$P = 0.009622$, *****$P = 0.00023$, ******$P = 0.000971$ in comparison with control tumour-bearing mice. The levels in the kidneys and lungs were recalculated on the basis of water content in these organs. Numbers in parentheses indicate the number of animals.

3.5. Effect of VAA on NO synthesis in culture of C3L5 cells

Endogenous production of NO by C3L5 cells because of eNOS expression was shown to stimulate tumour cell migration, invasiveness and angiogenesis [34,35]. To evaluate whether tumour growth and the metastasis-promoting activity of VAA resulted from additional NO induction by VAA in the C3L5 cells, we tested the effects of the lectin on $[NO_2^- + NO_3^-]$ accumulation in C3L5 cell cultures.

The basal level of $[NO_2^- + NO_3^-]$ in the cell media after 48 h of cultivation was 3.8±1.0 μM ($n=3$). The level significantly increased to 59.9±0.8 μM ($n=3$) in the presence of 10 μg/ml LPS and 500 U/ml IFN-γ, indicating an induction of iNOS in these cells, as previously reported by us [36]. The cells incubated for 48 h with VAA at concentrations of 1, 5, 10, 50, 100 or 1000 ng/ml generated $[NO_2^- + NO_3^-]$ ranging between 0.9 and 1.7 μM, which represent unchanged or reduced ($P<0.05$) levels. To test whether the minor reduction could be due to the toxicity of VAA, its carbohydrate-binding non-toxic B-subunit was added at the same concentrations to the C3L5 cells. This treatment also led to unchanged or decreased ($P<0.05$) levels ranging between 0.4–7.1 μM. Thus, additions of VAA and its B-subunit at immunomodulatory ~~as well as high~~ and higher concentrations failed to activate iNOS in the C3L5 cells.

4. Discussion

The principal objective of the present study was to test whether a biochemically purified plant lectin VAA given to mice at non-toxic immunomodulatory dosage provided additional therapeutic benefits in combination with IL-2 therapy. We utilised a well-characterised, eNOS expressing C3L5 murine mammary carcinoma model in C3H/HeJ mice to measure the effects of VAA and IL-2 therapy alone or in combination, on primary tumour growth, spontaneous lung metastasis, capillary leakage, NO production *in vivo*, and additionally the formation of the toxic NO-metabolite peroxynitrite, as indicated by the presence of immunoreactive nitrotyrosine in the kidney. Results of the VAA therapy alone were intriguing. They revealed that VAA given as biweekly injections for two weeks promoted the growth of primary tumours and stimulated the development as well as growth of spontaneous lung metastases. This occurred in spite of the inability of VAA to induce additional NO production by C3L5 cells *in vitro*. IL-2 therapy alone suppressed tumour growth and metastasis, but caused capillary leakage as evidenced by pleural effusion after the first round of therapy and fluid retention in the kidneys following both rounds of therapy. This was associated with sharp rises in NO levels in the serum (and pleural fluid) and organs (kidneys and lungs) after the first round, but a decline in the levels after the second round of therapy. Formation of peroxynitrite was evidenced by strong staining for nitrotyrosine in the renal tubules after both rounds of IL-2 therapy. Addition of VAA to IL-2 therapy provided no additional benefit nor detriment to IL-2 therapy as indicated by an absence of change in any of the above parameters in this tumour model.

Present findings of antitumour and antimetastatic effects of IL-2 therapy alone are a confirmation of our earlier studies with this tumour model utilising a different IL-2 regimen [3,11]. We have also confirmed the phenomenon of IL-2-induced capillary leakage in this tumour model, including the observation that mice surviving the pleural effusion after the first round do not develop pleural effusion after the second round of the IL-2 therapy [10–12]. In the present study, we noted that NO levels in the IL-2-treated mice rise sharply after the first round, but decline after the second round. We propose that pleural vessels can repair NO-mediated injury after withdrawal of high local NO levels. Thus a decline in the systemic and organ NO levels after the second round of IL-2 may explain the absence of pleural effusion. This decline is due likely to a negative feedback loop by which a sharp rise in NO levels can downregulate NOS expression, as well as NOS activity, as a protective mechanism against NO-mediated injury. This view is supported by a number of studies showing that high NO levels could inhibit both constitutive and inducible NOS activity. Feedback NOS inactivation appeared to be due to competition with oxygen (O_2) binding of NO to the haem iron prosthetic group [39] in association with a conserved tryptophan 409 residue [40]. Inhibitory action of NO on iNOS activity were demonstrated in macrophage cell lines J774 [41] and RAW 264.7 [42] using a set of NO donors, e.g. sodium nitroprusside and S-nitro-acetyl-penicillamine. Similarly, NO donors downregulated NO synthesis by the

Table 4
The level of nitrate + nitrite (μM) in mice after two rounds of therapy (experimental series I)

Animal group	Blood serum	Left kidney
1. Healthy mice	66.8±19.3 (10)	61.5±16.4 (10)
2. Control tumour-bearing mice	47.2±12.6 (10)*	52.9±30.1 (10)
3. IL-2 treatment	21.4±5.5 (7)**	29.7±12.2 (7)****
4. VAA treatment	47.0±6.6 (10)	41.6±13.4 (10)
5. Combined IL-2 and VAA treatment	17.0±6.1 (9)***	28.8±11.0 (9)*****

IL-2, interleukin 2; VAA, a galactoside-specific lectin from *Viscum album* L.
*$P=0.008308$, **$P=0.0000342$, ***$P=0.00000641$, ****$P=0.023962$, *****$P=0.018511$. The levels in the kidneys was recalculated on the basis of water content in this organ. Numbers in parentheses indicate number of animals.

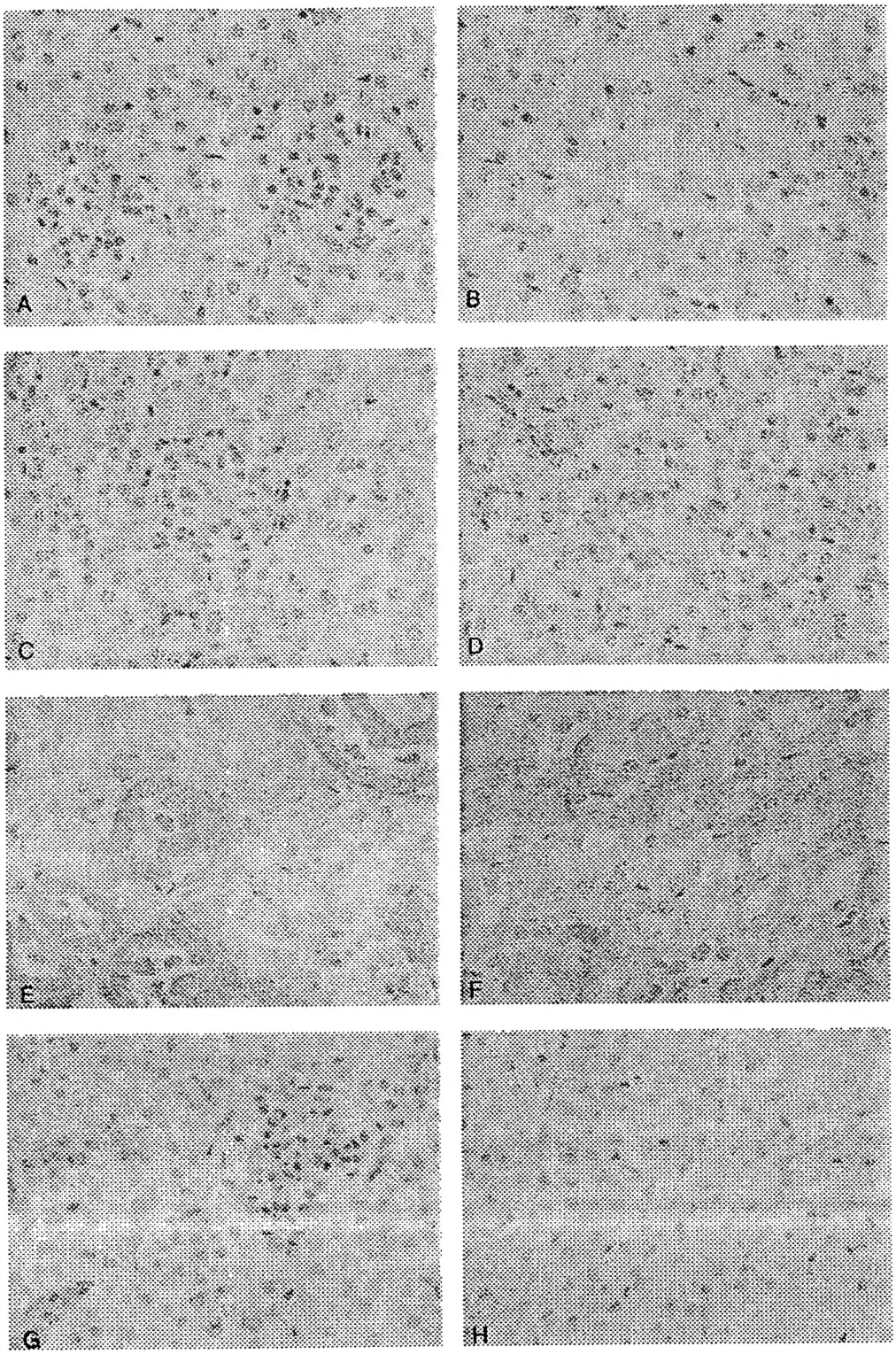

Fig. 2. Immunoperoxidase staining for nitrotyrosine in formalin-fixed paraffin-embedded kidney sections (×630) from C3L5 mammary adenocarcinoma-bearing C3H/HeJ female mice after two rounds of immunotherapy: (a, b) control tumour-bearing mice; (c, d) VAA-treated mice; (e, f) IL-2-treated mice; (g, h) negative control of IL-2-treated-mice (immunostaining with primary antibody was performed in the presence of 3-nitro-L-tyrosine). Left side (a, c, e, g) illustrates the cortical and the right side (b, d, f, h) the medullary regions of the kidneys. Kidney sections from VAA + IL-2-treated mice are not shown since their staining patterns were identical to those from IL-2-treated mice. Five sections from the right kidney of each animal ($n = 8$–10 mice/group) in the various groups were processed, and the pictures are representative for each group.

rat gastric myenteric plexus [43]. In other studies, NO has been shown to inhibit reversibly the activity of purified eNOS [44], as well as eNOS gene expression, in cultures of human coronary artery endothelial cells via a guanine monophosphate (GMP)-mediated mechanism [45]. In line with these observations, our data demonstrate for the first time the feedback regulation of NO production *in vivo* in the tumour-bearing mice treated with IL-2. Our results reveal that in spite of a decline in systematic NO levels after the second round of IL-2 therapy, kidneys exhibit significant capillary leakage, as opposed to the absence of pleural effusion. Thus pleural and renal capillaries may behave differently. It is possible that the latter is more sensitive to the injury resulting from peroxynitrite formation. Indeed, we have shown that in spite of a drop in renal NO levels, nitrotyrosine, a marker for peroxynitrite, was abundantly present in the kidneys after both rounds of IL-2 therapy.

In view of an increasing appreciation of the role of glycan chains of cellular glycoconjugates in cell signalling via interaction with endogenous lectins, research on plant lectins as diagnostic and therapeutic tools has attracted considerable interest [46,47]. Unfortunately, however, in recent times, popular alternative (complementary) modalities of human cancer therapy have frequently ascribed antitumour functions to mistletoe extracts, in spite of the lack of any clinical validation of such claims and documented opposing actions of lectin-induced cytokines *in vivo* [29,30]. The recent introduction of lectin-standardised extracts instead of preparations with variable lectin contents in the market is an attempted improvement to mimic the immunomodulatory capacity of the lectin. So far, case reports with extract application in the human remain discouraging, including some cases of stimulation of tumour growth [32,48]. As reviewed earlier, data derived from models of animal cancer remains conflicting, indicating beneficial [22–26] as well as detrimental [27,28] effects.

Our data demonstrate that the purified VAA promotes tumour growth and lung metastases in mice in the C3L5 mammary adenocarcinoma model. An important feature of this cell line is the constitutive expression of eNOS and induction of iNOS after stimulation with LPS and IFN-γ [36]. We had earlier shown that endogenous NO promoted tumour progression and metastasis in this tumour model by multiple mechanisms including stimulation of tumour cell migration, invasiveness and NO-mediated angiogenesis [34,35]. We have further shown that additional induction of iNOS in the presence of LPS and IFN-γ promotes invasiveness of these cells by an upregulation of matrix metalloproteases (MMP-2) [36]. However, VAA and its carbohydrate-binding B-chain failed to activate iNOS in the C3L5 tumour cells in contrast to the action of LPS + IFN-γ. Thus VAA-induced progression of tumour growth and metastasis in this cancer model cannot be attributed to iNOS induction with VAA. In contrast to the present finding with VAA, certain lectins from *Canavalia ensiformis* (Con A), *Canavalia brasiliensis*, *Pisum arvense*, *Dioclea grandiflora* were found to induce a significant accumulation of nitrite in the culture of unfractionated peritoneal cells from female BALB/c mice [49]. Furthermore, i.p. administration of two lectins from *Tricholoma mongolicum* to normal and sarcoma-bearing C57BL/6 mice primed peritoneal macrophages to produce nitrite and inhibited tumour growth most likely due to NO-mediated tumour cell cytotoxicity [50]. However, in many human tumours, in particular, cancers of the breast, oropharynx, lungs and the pancreas, increased NOS activity and expression in tumours is positively associated with tumour progression [51] indicating the risks of using NO-inducing agents in the clinic. In the present case, VAA stimulation of C3L5 tumour growth may be due to a direct stimulation of cell proliferation [31] or indirect stimulation by VAA-induced cytokines *in vivo* [30]. These possibilities remain to be investigated. While animal studies may not always predict the clinical outcome in the human, our results suggest that the use of VAA for cancer therapy in the clinic should be considered with caution. They also demonstrate that there is no advantage in using VAA as an immuno-adjuvant for IL-2-based immunotherapy.

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